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"DETERMINATION OF GLYCOSIDASES IN BABACO (*Vasconcellea* x Heilbornii cv. Babaco) PURIFICATION AND CHARACTERIZATION OF α-MANNOSIDASE FROM BABACO LATEX"

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DECLARATORIA

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M. Fernanda Reyes P.

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TABLE OF CONTENTS

TABLES

FIGURES

PAPER I

PAPER II

PAPERS

PAPER I. Fernanda Reyes, Hans Blom, Jan Carlsson, Jenny Ruales, **A screening of glycosidases in babaco (*Vasconcellea x Heilbornii* cv. Babaco)**

PAPER II. Hans Blom, Fernanda Reyes, Jenny Ruales, Jan Carlsson, **Purification and characterization of an α-mannosidase from the tropical fruit babaco *(Vasconcellea* x *Heilbornii* cv. babaco)**

ABBREVIATIONS

AC	Affinity chromatography
BSA	Bovine serum albumin
CIP	Cleaning in place
CM	Carboxymethyl
Con A	Concanavalin A
DEAE	Diethylaminoethyl
DNA	Deoxyribonucleic acid
EC	Enzyme Comission
EDTA	Ethylenediamine tetraacetic acid
FPLC	Fast performance liquid chromatography
GF	Gel filtration
HIC	Hydrophobic interaction chromatography
HMW	High molecular weight
HP	High performance
IEC	Ion exchange chromatography
IMAC	Immobilized metal ion chromatography
mosl	Meters over the sea level
MW	Molecular weight
PEG	Polyethylene glycol
PG	Prep grade
pI	Isoelectric point
pNPA	p-nitrophenyl α-L-arabinopyranoside
pNPAG	p-nitrophenyl N-acetyl β-D-acetyl-glucosaminide
pNPF	p-nitrophenyl β-D-fucopyranoside
pNPG	p-nitrophenyl-β-D-glucopyranoside
pNPGal	o-nitrophenyl β-D-galactopyranodide
pNPM	p-nitrophenyl α-D-mannopyranoside
RPC	Reverse phase chromatography
SDS-PAGE	Sodium dodecyl sulfate polyacrylamide gel electrophoresis

UNITS

ºC	Celsius degree
cm	centimeter
Da	Dalton
g	gram
kg	kilogram
kDa	kilodalton
mg	milligram
ml	milliliter
mm	millimeter
mM	milimolar
M	molar
nm	nanometer
µg	microgram
µl	microliter
µm	micrometer
µmol	micromol
U	Enzyme unit

RESUMEN

El babaco (*Vasconcellea* x Heilbornii cv. Babaco) es una fruta nativa del Ecuador, que además de presentar excelentes propiedades sensoriales es rica en enzimas como proteasas, lipasas, glicosidasas y peroxidasas. Exuda un látex comparable al obtenido en otros frutos como la papaya (*Carica papaya*), y existen algunos estudios sobre la purificación y caracterización de glicosidasas en el látex de la papaya.

El objetivo de esta investigación fue determinar las glicosidasas en las diferentes partes del babaco: cáscara, pulpa y látex. Se determinó también la actividad de las glicosidasas en el látex de papaya para compararlo con el látex de babaco. Se purificó y caracterizó la α-manosidasa en el látex de babaco liofilizado, aplicando técnicas de cromatografía de afinidad, cromatografía de intercambio iónico, filtración en gel, y electroforesis.

Las glicosidasas ensayadas en el babaco fueron: α-arabinosidasa, β-glucosidasa, β-fucosidasa, β-galactosidasa, acetil β-glucosaminidasa y α-manosidasa, esta última tiene la mayor actividad en el látex de babaco liofilizado. Las actividades de las otras enzimas glicosídicas fueron relativamente bajas.

Se optimizó el método de extracción de las enzimas glicosídicas en base al pH y concentración del búffer, siendo las condiciones óptimas de extracción 30 mg/ml de muestra liofilizada (cáscara, pulpa o látex) en 20mM Tris pH 7.5 Se desarrolló el método más efectivo para determinar la actividad de la α-manosidasa con respecto a la temperatura y tiempo de ensayo, 50ºC y 30 minutos de incubación, con una concentración de sustrato de 5 mM en 200 mM de acetato de sodio a pH 4.5.

Finalmente, se purificó la α-manosidasa del látex de babaco liofilizado utilizando cromatografía de intercambio iónico (Q-Sepharose), cromatografía de afinidad (Con A Sepharose) y filtración en gel (Superdex 200). Se caracterizó la enzima purificada en base a su temperatura y pH óptimos, 50ºC y 4.5 respectivamente, peso molecular

(230 kDa), punto isoeléctrico (5.85 – 6.55). Se determinó el efecto de inhibidores (Cu, Mn) y activadores (Zn), estabilidad y composición en base a los aminoácidos.

ABSTRACT

Babaco (*Vasconcellea* x Heilbornii cv. Babaco) is a native fruit from Ecuador that presents excellent sensorial properties but also is considered as a good enzymatic source. It is rich in different enzymes mainly proteases, lipases, peroxidases and glycosidases. Babaco when green (unripe) exudates a white milky substance known as "latex" that is comparable with the latex from other fruits like papaya (C*arica papaya*).

The objective of the present thesis work was to determine the glycosidases in different parts of babaco: peel, pulp and latex. The glycosidic activities were determined also in papaya latex in order to make a comparison with babaco latex.

The α-mannosidase in freeze-dried babaco latex was purified and characterized, by techniques such as affinity chromatography, ion-exchange chromatography, gel filtration and electrophoresis.

A screening for glycosidases in different parts of babaco was made. The tested enzymes were: α-arabinosidase, β-glucosidase, β-fucosidase, β-galactosidase, acetyl β-glucosaminidase and α-mannosidase. This last enzyme was found mainly in freeze-dried babaco latex where it presented the highest activity of the studied enzymes. The activities of the other glycosidases were significantly lower.

Thus, the main aim has been the development of optimal conditions such as pH and buffer concentration for an efficient extraction of glycosidases. The final conditions were 30 mg/ml of freeze-dried sample (peel, pulp or latex) in 20 mM Tris pH 7.5. A convenient and reliable method for the α-mannosidase activity measurement was also developed. The optimum temperature and pH were determined. The selected conditions were 50ºC and 30 minutes of incubation, with a substrate concentration of 5 mM in 200 mM of sodium acetate pH 4.5. Finally the α-mannosidase of the freeze-dried babaco latex was purified using a three step procedure: ion-exchange chromatography (Q-Sepharose), affinity chromatography (Con A Sepharose) and gel filtration (Superdex 200). The temperature and pH optima for the enzyme were found to be 50ºC and 4.5 respectively. Its molecular weight and isoelectric point were determined to 230 kDa and 5.85 – 6.55 respectively. The effects of some inhibitors (Cu, Mn), and one activator (Zn) were examined. Finally was also the stability of the mannosidase studied and its amino acid composition determined.

1. INTRODUCTION

The use of enzymes in industry is increasing every day because of the many benefits that this brings especially to the environment and for optimization of the many processes in e.g. food industry. Enzymes are found everywhere in living organisms such as animals, plants and microorganisms. Scientists are continuously searching for new ones and try to find reliable and easy method for their purification and then they study their properties in order to find the optimum conditions to make them work in different applications.

Babaco (V*asconcellea x Heilbornii* cv. babaco) which is a fruit native from the mild climate zones of Ecuador was previously known as *Carica pentagona.* The babaco production has increased considerably during the last years because of good production yields and excellent sensorial properties, but also because babaco is considered as a good enzymatic source. It is rich in different enzymes like proteases, lipases, glycosidases and peroxidases.

The following study had been focused on the babaco glycosidases, which are sugar degrading enzymes with their primary role in plants and animals is the hydrolysis of glycosides. Glycosidases are very important in food industry because they enhance the flavor of some products because of the polysaccharide degradation. They are used in brewery and bakery and they are known also to participate in fruit ripenening and plant defence (Kirk and Othmer,1963; Waln and Poulton,1987; Sorensen, Kragh et al.,2004)

The enzyme α-mannosidase is present in freeze-dried babaco latex. Its purification and characterization studies will give information about this enzyme and its properties. Many physiological roles for plant α-mannosidases including participation in fruit ripening, removal of mannose residues from storage glycoproteins upon seed germination and processing of cellular glycoproteins other than reserve proteins are known (Waln and Poulton,1987).

For the purification of an enzyme, chromatography is by far the most important method. Different separation principles, like ion exchange, size exclusion and affinity interactions are utilized in the various methods. Electrophoretic methods are also employed for the determination of the enzyme purity, its molecular weight and isoelectric point. The enzyme is characterized by determining its optimum pH and temperature, stability, specificity, how its activity is affected by activators and inhibitors and its amino acid composition (Scopes,1987).

2. RESEARCH OBJECTIVES

2.1. General objective

The general objective was to study glycosidic enzymes in babaco and try to purify and characterize the α-mannosidase by chromatographic techniques and electrophoresis.

2.2. Specific objectives

1. To optimize the method of extraction of the glycosidases from different parts of the babaco fruit: peel, pulp and latex.

2. To develop an efficient and reliable method to determine the glycosidic activities in babaco.

3. To purify the α-mannosidase of the babaco latex and characterize the purified enzymes activity as a function of temperature, pH, presence of activators and inhibitors and determine its molecular weight, isoelectric point and amino acid composition.

3. LITERATURE REVIEW

3.1. BABACO (*Vasconcellea x Heilbornii* cv. Babaco)

3.1.1 General Aspects

Babaco is a natural hybrid between *Vasconcellea stipulata* known as "toronche" and *Vasconcellea cundinamarcensis* known as "chamburo". Babaco is native from Loja Province, a place at the South part of Ecuador; nowadays it is cultivated principally in the subtropical valleys at 1500 – 2500 meters over the sea level (mosl) in places like Atuntaqui, Tumbaco, Patate, Baños, Gualaceo, Loja (Merino,1989).

Babaco is a small herbaceous shrub that grows to about one to three meters long. It has an erect softwood trunk lined with leaf scars. The plant rarely branches but shoots often appear around the base. The thickness of the trunk is associated with the vigor of the plant. The moderately large, palmate, green leaves have prominent ribs and veins and are on long hollow petioles that radiate from the trunk. The flowers are formed on the newly developing trunk during the growth phase of the tree. They are very attractive and have white yellowish color and they arise axially from every leaf. The flowers are all female. The fruits set and grow immediately after flowering. It is a seedless fruit. They are distinctly five-sided, rounded at the stem end and pointed at the apex. Annual yields are 60 to 80 fruits per plant (Guerrero and Castro,1999). Fruits ripen in progression from the lower fruits, usually the heaviest, to those higher up the trunk (Figure 1).

Babaco fruit is very juicy, slightly acidic and low in sugar. It has a very special flavor described as a cross between pineapple, lemon and papaya (Villarreal, Dhuique-Mayer et al.,2003). The fruit weight is around 0.8 kg to 2.2 kg. Its length can reach 38 cm and its diameter 14 cm. When the fruit is ripe the peel is gold yellow and the pulp has a light yellow color. The peel is very soft and thin, and very easy to remove (Albuja,2001)

Figure 1. Babaco tree

The fruit can be directly consumed when it is fully ripe but it is also possible to produce pulp, jam or dehydrated fruit that can be use in the elaboration of ice creams, yogurts and marmalades.

When the fruit is green it exudates a white substance, containing a number of enzymes, known as "latex" that is comparable with the latex from other fruits like papaya (C*arica papaya*) which has enzymatic activity.

Besides being an exotic fruit that presents excellent sensorial properties, babaco is also considered as a good enzymatic source. It is rich in different enzymes mainly proteases, lipases, peroxidases and glycosidases (Marcillo,2005). The extraction and purification of these enzymes could have a potential industrial application.

3.1.2 Babaco latex

Babaco latex is a white milky substance that is comparable with papaya latex (*Carica pentagona)* a close relative to babaco. It is present in leaves, trunk and fruits of the plant. Its principal constituents are water, proteins, lipids and carbohydrates (Table 1) (Ruales,2003; Marcillo,2005).

Fresh latex has a high pH and its water activity is also high, so it is very sensible to microorganisms then the importance of apply a proper drying method to facilitate its conservation, transportation and storage and to protect its enzymatic activity (Marcillo,2005).

Table 1. Babaco latex characterization

Property	Value
pH	6.01
Humidity (%)	80.47
Proteins (%)*	12.34
Lipids (%)*	1.36
Carbohydrates (%)*	5.83

* % in base of dry matter

Source: Ruales *et al., 2003*

Babaco latex is extracted from totally green babacos which are 4 to 7 months, around 16 to 23 cm length and level 0 according to the babaco color chart, in this age the yield of latex and enzymatic activity is optimum (Villarreal,2001).

The method for collecting babaco latex is very similar to the one used for collecting latex from papaya. Tapping or "milking", as it is called the latex extraction, has to be done from first light until an hour after sunrise. Normally babaco latex was collected from 8 to 9:30 a.m. It is not advisable to tap for latex in very hot dry, very cold or windy conditions as the flow of latex will decrease (Moore,1980). There may be three or four fruits ready for tapping on a single tree at any time.

The first step for milking is to clean the surface of the fruit with a piece of cloth to remove dust and all small impurities. Make up 3 superficial cuts, in 3 of the 5 faces of the fruit, no more than 1-2 mm deep with a stainless steel blade. Let the latex run to the lowest point of the fruit and drop on to the collecting sterilized recipient. Scrape off the latex which coagulates on the fruit and clean again the fruit with a cloth, leave the fruits in the tree until they are ripe for harvest (Figure 2).

Figure 2. Method for collecting babaco latex

The collected latex has to be dried the same day of tapping; there are different methods to dry latex, sun-drying which gives a low quality product, a hot air drier which is cheaper and the product keeps the quality, and freeze drier that is more expensive but the final product has a very good quality (Marcillo,2005). The latex used in this study was frozen at -20ºC and then freeze-dried in a LYOVAC GT2 lyophilizer. Freeze-dried

latex was ground and stored in plastic containers at 0ºC until the characterization of enzymes was done (Figure 3).

Figure 3. Lyophilized babaco latex

3.2 ENZYMES

An enzyme is a specialized protein that acts as a biological catalyst. A catalyst is something that speeds up a chemical reaction. Therefore, enzymes are biological molecules which increase the rates of chemical reactions (Stryer, Berg et al.,2002).

Enzymes are highly specific and are the most efficient catalysts known. Enzymes accelerate (often by several orders of magnitude) chemical reactions in the cell that would proceed imperceptibly or not at all in their absence. They are not modified or consumed during the reaction but they loose their activity with heat, chemical agents or extreme conditions of pH or temperature (Nelson,2004).

Most enzymes demonstrate great specificity, reacting with only one or a small group of closely related chemical compounds that are known as substrates; several enzymes are sometimes required for efficient catalytic function. Some enzymes depend on the presence of coenzymes for their function. For the enzyme to continue to be effective, its three-dimensional molecular structure must be maintained (Voet and Voet,1990).

Enzymes occur naturally in most raw materials of biological origin. They are present in animals, plants and microorganisms. Nowadays the use of enzymes in industry is very important, they optimize the process for the production of detergents, additives, food products, beer and wine, chemical and pharmaceutical products and others.

In some cases, for example the production of yogurt, cheese, beer and wine needs fermentation which is not possible without the enzymes present in microorganisms and which are part of the process. However, some processes in food industry can be performed using isolated and purified enzymes from microorganisms, plants or animal tissues (Reed,1975) .

The enzymatic technology is an alternative for industries to develop high quality products, make an optimal use of the raw materials, accelerate processes, minimize waste and protect the environment by substituting traditional chemical or technical solutions for enzymes (Sorensen, Kragh et al.,2004).

3.2.1 Glycosidases

Glycosidases (E.C. 3.2.1.) are sugar degrading enzymes with their primary role in plants and animals in the hydrolysis of glycosidic bonds between monosaccharide units of a polysaccharide. Each type of glycosidase is often a group of enzymes that hydrolyze the same type of bond, but they have different specificity. The affinity of the enzyme for a particular substrate is dependent on the nature of the enzyme source (Woodward and Wiseman,1982).

Glycosidases are also known to be involved in the biosynthesis of the oligosaccharide chains and quality control mechanisms in the endoplasmic reticulum of the N-linked glycoproteins. Inhibition of these glycosidases can have profound effects on quality control, maturation, transport, and secretion of glycoproteins and can alter cell-cell or cell-virus recognition processes (Asano,2003)

Enzymatic hydrolysis of complex carbohydrates containing α– or β– glycosidic bonds is very important in nutrition and in several technological processes (Sorensen, Kragh et al.,2004). Glycosidases are very important in food industry as they enhance the flavor of some products because of the polysaccharide degradation. They are used to improve the shelf-life of bakery products, clear beer, produce glucose, fructose or dextrins, hydrolyze lactose, modify food pectins or improve processes (Kirk and Othmer,1963; Sorensen, Kragh et al.,2004).

According to the database of the International Union of Biochemistry and Molecular Biology that had proposed the enzyme nomenclature based on the EC numbers, there are 161 glycosidases (EC 3.2.1.1 to EC 3.2.1.161) that hydrolyze specific glycosidic bonds (Anonymous,2006). The glycosidic enzymes that were tested for activities in babaco were chosen following some studies of glycosidases that had been done in papaya (Hartmann-Schreier and Schreier,1986; Hartmann-Schreier and Schreier,1987). The enzymes that present some activity in babaco fruit are presented in Table 2, with their synthetic substrate and the corresponding EC number.

Table 2. List of glycosidic enzymes tested in babaco latex

Enzyme	***Other name***	***Substrate***	***EC number***
α-arabinosidase	α-N-arabinofuranosidase	p-nitrophenyl α-L-arabinopyranoside (pNPA)	EC 3.2.1.55
β-glucosidase	Cellobiase, emulsin	p-nitrophenyl-β-D-glucopyranoside (pNPG)	EC 3.2.1.21
β-fucosidase		p-nitrophenyl β-D-fucopyranoside (pNPF)	EC 3.2.1.38
β-galactosidase	Lactase	o-nitrophenyl β-D-galactopyranoside	EC 3.2.1.23
Acetyl -β-glucosaminidase	Chitinase	p-nitrophenyl N-acetyl β-D-glucosaminide (pNPAG)	EC 3.2.1.14
α-mannosidase	α-D-mannoside mannohydrolase	p-nitrophenyl α-D-mannopyranoside (pNPM)	EC 3.2.1.24

3.2.1.1 α-Mannosidase

α-Mannosidase (EC 3.2.1.24) is a type of glycosidase that participates in the hydrolysis of terminal, non-reducing α-D-mannose residues in α-D-mannosides (Anonymous,2006; Anonymous,2006).

The reaction with the substrate p-nitrophenyl-α-D-mannopyranoside (pNPM) is presented in Figure 4.

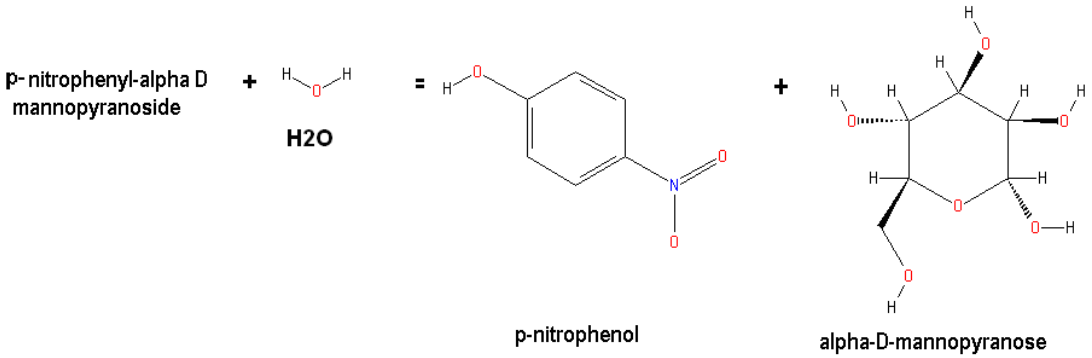

Figure 4. pNPM Reaction

In the presence of active enzyme the substrate releases p-nitrophenol as product, a yellow colored substance that can be monitored at 405 nm. After incubation the pH has to be increased in order to improve detection (increases the color intensity). The enzyme activity is related to the absorbance value measured at 405 nm.

α-Mannosidases are widely distributed in plants, bacteria and animals. Functionally the enzyme is implicated, in conjunction with other glycosidases, in the degradation of polysaccharides, glycoproteins and other glyco-conjugates during seed germination to provide the nutrient and energy requirements of the growing seedling (Niyogi and Singh,1988). Many physiological roles for plant α-mannosidases include participation in fruit ripening, removal of mannose residues from storage glycoproteins upon seed germination and processing of cellular glycoproteins other than reserve proteins (Waln and Poulton,1987)

In freeze dried babaco latex, α-mannosidase presents the highest activity. It was therefore interesting to try to purify it to be able to determine its structure and properties.

3.3 PURIFICATION OF ENZYMES

The development of techniques and methods for the separation and purification of biological macromolecules like enzymes (proteins) is very important for bioscience and biotechnology, for medical and industrial purposes.

The purification of an enzyme is not an easy process and there are a few crucial considerations that have to be taken like the selection of a suitable source for the enzyme, the availability and cost of the raw material, the stability of the enzyme, the presence of interfering activities and proteins, and difficulties in handling a particular raw material (Janson and Rydén,1998). It is also important to perform a literature survey to see what had previously been done with the chosen enzyme and source.

Of the different sources for enzymes available, animal tissues are more expensive and more complex, although the absence of cell walls makes homogenization easy, but fat present in such tissues can often cause problems. Yeast and bacteria have tough cell wall so they are more difficult to homogenize. Plants contain large amounts of fibrous bulk material and often polyphenols and pigments that may interfere in the purification process. Bacteria have a relatively high content of nucleic acids which have to be removed (Janson and Rydén,1998).

The aim of a protein purification process is three-fold: to remove unwanted contaminants, to concentrate the desired component, and to transfer the protein to an environment where it is stable and in a form ready for the intended application.

Enzyme purification usually consist of several steps that include the preparation of the crude extract that results in a clarified and concentrated protein solution, application of chromatographic techniques, where the majority of the contaminating proteins and other unwanted substances are removed, and finally a polishing step, where the remaining

trace impurities like aggregates and modified forms of the protein itself are removed (Scopes,1987).

Electrophoretic techniques are used for analytical separations, to evaluate the purity of the enzyme and to determine its molecular weight and isoelectric point.

3.3.1 Extraction Methods

The aim of the extraction is to enrich the desired protein in one phase, either as a precipitate, partioned to an aqueous polymer phase, or adsorbed to a solid phase medium. The goal is to prepare the sample for high resolution chromatographic step (Scopes,1987).

3.3.1.1 Extraction Medium

The first thing to consider is the extraction solution that needs to have the conditions in which the protein of interest is stable (Janson and Rydén,1998). These conditions are:

pH.- To get the most efficient extraction a pH which gives the maximum activity or maximum stability of the protein is usually selected. The use of extreme pH values is sometimes very efficient and is acceptable for some enzymes without causing excessive denaturation.

Buffer salts.- Most of the proteins are soluble at moderate ionic strengths, around 0.05 to 0.1 and values in this interval are chosen if the buffer capacity is sufficient. Some suitable salts are sodium acetate, sodium bicarbonate, sodium citrate, ammonium acetate, Tris-chloride, sodium phosphate and Tris-phosphate.

Detergents and chaotropic agents.- Sometimes in the extractions the protein can be bound to membranes or particles or is aggregated due to its hydrophobic character. These hydrophobic interactions should be reduced by using detergents that can have a mild or nondenaturing effect on proteins like Triton X-100, Nonidet P-40, Lubrol PX, Octyl glucoside or detergents which have a strong denaturing effect on protein like

Sodium dodecyl sulfate (SDS). The use of detergents is restricted to the extraction medium. Also some chaotropic agents can be used like urea and guanidine hydrochloride, or moderately hydrophobic organic compounds such as ethylene glycol.

Reducing agents.- Some proteins have exposed thiol groups, which might be oxidized in the purification process. Thiol groups can be protected by reducing agents such as 1,4-dithioerythritol (DTE), 1,4.dithiothreitol (DTT) or mercaptoethanol. Low concentrations (10-25 mM) of these agents are sufficient to protect thiols without reducing internal disulfides.

Chelator or Metal ions.- The presence of heavy metals can be negative for the activity of the protein because they can enhance the oxidation of thiols by molecular oxygen and they can form complexes with specific groups that can cause problems. To avoid this, the heavy metals they can be trapped by chelating agents such as ethylenediamine tetraacetic acid (EDTA) in the concentration range around 10 to 25 mM. It is best to add the disodium salt of EDTA before final pH adjustment. The chelating capacity of EDTA increases with increasing pH.

Proteolytic inhibitors.- Proteases are the most serious threats for protein stability. The simplest way to avoid proteolytic degradation is to work fast at cold conditions. An alternative is to add protease inhibitors like Diisopropyl fluorophosphates (DFP) or Phenylmethylsulfonyl fluoride for serine proteases, EDTA for metal-activated proteases, especially in connection with the extraction step. Sometimes it is enough to adjust the pH to a value at which the proteases are inactive, but where the stability of the desired protein is maintained.

Bacteriostatics.- In order to avoid bacterial growth it is necessary to filter the solution of the extraction by sterilized filters that can be of different pore size (0,22 μm or 0.45 μm). This is a routine step in the laboratory and is applied to every solution used. It will also reduce the risk of bacterial growth in the chromatographic columns. Buffers at pH 3 and below or 9 and above usually prevent bacterial growth, but may occasionally allow growth of molds.

3.3.1.2 Disintegration Method

Different techniques are applied depending on the type of cell to be disintegrated; there exit techniques which are gentle, moderate or vigorous to the cells. Table 3 shows a number of techniques used for cell disintegration.

Table 3. Cell Disintegration Techniques

Technique	Example	Principle
Gentle		
Cell lysis	Erythrocytes	Osmotic disruption of cell membrane
Enzyme digestion	Lysozyme treatment of bacteria	Cell wall digested, leading to osmotic disruption of cell membrane
Chemical solubilization /autolysis	Toluene extraction of yeast	Cell wall (membrane) partially solubilized chemically; lytic enzymes released complete the process
Hand homogenizer	Liver tissue	Cells forced through narrow gap, rips off cell membrane
Mincing (grinding)	Muscle, etc.	Cells disrupted during mincing process by shear forces
Moderate		
Blade homogenizer (Waring type)	Muscle tissue, most animal tissues , plant tissues	Chopping action breaks up large cells, shears apart smaller ones
Grinding with abrasive (e.g. sand, alumina)	Plant tissues, bacteria	Microroughness rips off cell walls
Vigorous		
French press	Bacteria, plant cells	Cells forced through small orifice at very high pressure; shear forces disrupt cells
Ultrasonication	Cells suspensions	Microscale high-pressure sound waves cause disruption by shear forces and cavitation
Bead mill	Cells suspensions	Rapid vibration with glass beads rips cell walls off
Manton-Gaulin homogenizer	Cell suspensions	As for French press above, but on a larger scale

Source: (Samuelsson,2006)

3.3.2 Precipitation

Precipitation is a method used as a preliminary step for crude fractionation or to obtain clear or more concentrated sample solutions. Precipitation of a protein in an extract may be achieved by adding salts (ammonium sulfate), organic solvents (ethanol, 2-propanol, acetone), or organic polymers (PEG). An adjustment of the pH can also be used to precipitate a protein, and it is the simplest and inexpensive way. Keeping the salt concentration constant and varying the temperature is another way of fractionating a protein solution (Janson and Rydén,1998).

Most of these precipitations produce a non-crystalline product which may constitute aggregates of several molecular species or contain significant amounts of occluded solvent or absorbed salts. Precipitates are therefore impure compared to crystals (Ward,1991).
Precipitation of proteins by "salting out" can serve to both purify and concentrate the particular protein fraction.

The four following variables are usually kept under control: pH, temperature, protein concentration and the ionic strength. Low temperature during the precipitation process is often necessary to avoid protein denaturation. At constant ionic strength, the solubility of a protein is the least at its isoelectric point and increases on either side of this value. At low concentration the protein solubility is increased with increasing salt concentrations, a phenomenon known as "salting in". When the ionic strength is further increased, the solubility of proteins start to decrease, and they will eventually precipitate (salting out) (Ward,1991).

The most common salt used for protein precipitation is ammonium sulfate because of its high solubility, its lack of toxicity to most proteins, and since it's cheaper than other salts, and sometimes acts as a protein stabilizer.

The precipitation of a protein by increasing the temperature normally causes irreversible protein denaturation. This can be used to remove contaminants of heat labile proteins from a relatively thermo stable protein product. Heat treatment is also used to inactivate virus, proteases and to precipitate DNA.

After precipitation, centrifugation has to be used as a routinely step in protein purification laboratory to recover precipitates.

3.3.3 Concentration – Membrane process

Concentration of a protein solution can be done by membrane technology (Samuelsson,2006). The different types of membrane filtration are classified by the membrane pore size. Microporous membrane filters have a minimum pore size, in the range of 0.1 -10 microns in diameter. They retain particles like whole cells or cell debris (Blanch and Clark,1996). This technique is known as microfiltration and is very much used in bioprocesses, for clarification and to achieve sterility (Docksey,1986). Other techniques are reverse osmosis and ultrafiltration which have recently been introduced and are based on membranes with smaller pores. The upper size limit of molecules for reverse osmosis membranes is 500-1000 Da and for ultrafiltration membranes is 1000-1000 000 Da.

Membranes are produced from a range of different polymers, depending on biological and chemical parameters, mechanical and thermal compatibility and pore size distribution.

Organic membranes like cellulose, have very limited chemical and thermal compatibility, are biodegradable, have narrow cut off ranges and are difficult to produce. Ceramic membranes were introduced recently, and they have rigid porous matrix, and they have the advantage that they operate in a wide range of pH (1-13), can tolerate temperatures up to 140ºC and that low pressure differentials are observed across the filter (Ward,1991).

Microfiltration is useful in the concentration of protein solutions in the early stages of purification, while ultrafiltration is used for the purification, concentration and desalting of proteins and other molecules.

Cross-flow systems are used to alleviate the build-up of material that can cause filter blocking and/or clogging during conventional filtration. The liquid on the upstream side

can be circulated tangential to or across the filter surface; this serves to sweep the membrane clean of deposited particles (Blanch and Clark,1996).

3.3.4 Chromatographic Techniques

Separation by chromatography depends on the differential partition of proteins between a stationary phase and the mobile phase. The stationary phase is the medium or the adsorbent that normally is packed into a vertical column of plastic, glass or stainless steel; and the mobile phase is the buffer solution that is pumped through this column.
A batch adsorption can be done as an alternative to the stationary phase and consist in stirring the protein solution with the adsorbent and then pouring the slurry onto an appropriate filter and make the washings and desorptions on the filter (Janson and Rydén,1998).

Different chromatographic techniques can be applied to purify a protein depending on the separation principles. The most important separation principles and the corresponding types of chromatography are listed in Table 4.

Table 4. Chromatography in Biotechnology

Separation Principle	Type of chromatography
Size and shape	Size exclusion chromatography (Gel Filtration-GF)
Net charge	Ion exchange chromatography (IEC)
Isoelectric point	Chromatofocusing
Hydrophobicity	Hydrophobic interaction chromatography (HIC) Reverse phase chromatography (RPC)
Biological function	Biospecific affinity chromatography
Antigenicity	Immunosorption
Carbohydrate content	Lectin affinity chromatography
Content of free –SH	Covalent chromatography (chemisorption)
Metal binding	Immobilized metal ion affinity chromatography (IMAC)
Miscellaneous	Dye ligand affinity chromatography

Source: (Janson,2006)

3.3.4.1 Ideal Matrix

The ideal matrix or medium for chromatography of proteins should be chemically and physically stable and resistant in order to withstand extreme conditions during derivatization and maintenance, and be rigid enough to allow high linear flow rates (5 cm/min or more) in columns packed with particles of diameters down to a few microns. Other properties of an ideal base matrix are (Janson,2006):

- Macroporous + large surface/volume ratio
- Hydrophilic and inert matrix
- Easily to derivatize
- Easily to repack in any column size
- Cleaning in place (CIP) compatible
- Favorable cost/performance ratio

Many different materials are used for chromatography mediums: Inorganic materials such as porous silica, controlled pore glass and hydroxyapatite; synthetic organic polymers like polyacrylamide, polymethacrylate and polystyrene/divinyl benzene; polysaccharides such as cellulose, dextran and agarose; composite materials like silica/dextran (starch, agarose), polyacrylamide/dextran (agarose) and agarose/dextran.

3.3.4.2 Order of chromatographic steps

There are a number of practical rather than theoretical reasons why one should choose certain chromatographic techniques for the early steps and others for the final steps of a protein purification process. The following parameters have to be kept in mind when planning the separation strategy.

1. The sample volume
2. The protein concentration and viscosity of the sample
3. The degree of the purity of the protein product
4. The presence of nucleic acids, pyrogens and proteolytic enzymes in the sample
5. The ease with which different types of adsorbents can be washed free from adsorbed contaminants and denatured protein.

The logical sequence of chromatographic steps would be to start with more “robust” techniques that combine a concentration effect with high chemical and physical

resistance and low material cost. It is better to avoid buffer changes and concentration steps. For example, it is recommended to start with an ion exchange chromatography because the eluted peaks, regardless of the ionic strength, can be applied to a gel filtration column. This also will work as a desalting step, which means that the buffer used for the gel filtration should be chosen so as to allow direct application of the eluted peaks to the next chromatographic step.

3.3.4.3 Size Exclusion Chromatography

Size exclusion chromatography (SEC) is also known as gel filtration (GF), the separation mechanism is based on partitioning of the proteins between two liquid phases, one stationary inside the gel particles and one mobile making up the void volume between the particles (Janson and Rydén,1998).

The separation depends only in differences in the sizes and shapes of the proteins and the pore size distribution of the three-dimensional network of the gel materials used for the chromatography. Molecules elute in order of size, the largest molecules come first and the smallest ones come last.

It is possible to determine the molecular weight of the protein by gel filtration if the column is calibrated with standards of proteins with the same general solvatization and asymmetry as the sample protein, an assumption that is reasonably valid for globular proteins as noted (Scopes,1987).

The technique can be applied in two different ways (AmershamBiosciences,2002):

1. Group separations: the components of a sample are separated in two major groups according to size range. A group separation can be used to remove high or low molecular weight contaminants or to desalt and exchange buffers.
2. High resolution fractionation of biomolecules: the components of a sample are separated according to differences in their molecular size. High resolution fractionation can be used to isolate one or more components, to separate monomers from aggregates, to determine molecular weight or to perform a molecular weight distribution analysis.

Gel filtration can also be used to facilitate the refolding of denatured proteins by careful control of changing buffer conditions.

To perform a separation, gel filtration medium is packed into a column to form a *packed bed.* The medium is a porous *matrix* in the form of spherical particles that have been chosen for their chemical and physical stability, and inertness. The packed bed is equilibrated with buffer which fills the pores of the matrix and the space in between the particles. The samples are eluted isocratically and there is no need to use different buffers during the separation. However, a wash step using the running buffer is usually included at the end of a separation to facilitate the removal of any molecules that may have been retained on the column and to prepare the column for a new run (AmershamBiosciences,2002).

Sample volumes are expressed as a percentage of the total column volume. For group separations sample volumes up to 30% of the total column volume can be applied. For high resolution fractionation a sample volume from 0.5-4% of the total column volume is recommended, depending on the type of medium used. To increase the capacity of a gel filtration separation samples can be concentrated. Concentrations above 70 mg/ml protein should be avoided as viscosity effects may interfere with the separation.

Sample dilution happens because diffusion occurs as sample passes through the column. In order to minimize sample dilution use a maximum sample volume that gives the resolution required between the peaks of interest.

In order to choose the appropriate medium, three main factors have to be considered (AmershamBiosciences,2002):

1. The aim of the experiment (high resolution fractionation or group separation)
2. The molecular weights of the target proteins and contaminants to be separated.
3. The final scale of purification.

Different types of mediums for gel filtration are available:

Superdex is the first choice for high resolution, short run times and high recovery.

Sephacryl is suitable for fast, high recovery separations at laboratory and industrial scale.

Superose offers a broad fractionation range, but is not suitable for large scale or industrial scale separations.

Sephadex is ideal for rapid group separations such as desalting and buffer exchange. It is used at laboratory and production scale, before, between or after other chromatography purification steps.

The medium is chosen depending on the objectives of the experiment.

3.3.4.4 Ion Exchange Chromatography

Ion exchange chromatography (IEC) is one of the most important and most general liquid chromatography methods for protein purification. Some of its characteristics are: it is easy to apply, very widely applicable, it can give very good resolution, it often gives high yields of active material, it is a concentrating technique and can be easily controlled to meet specific needs (Janson and Rydén,1998).

Separation in IEC depends on the difference in the protein surface charges. An ion exchanger is the chromatographic medium that has an opposite charge to the protein. The strength of the binding depends on the surface charge density of the protein and on the ion exchanger; the more highly charged the protein and the ion exchanger, the stronger the interaction.

Ionically bound proteins are eluted differentially from the ion exchanger, either by increasing the concentration of ions which compete for the same binding sites on the ion exchanger by the addition of simple salt, or by changing the pH of the eluent so that the proteins loose their charges by titration (Scopes,1987).

Five main stages describe the principle of IEC (AmershamBiosciences,2002):

1. *Equilibration*, in which the ion exchanger is brought to a starting state, in terms of pH and ionic strength, which allows the binding of the desired solute molecules. The exchanger groups are associated at this time with exchangeable counter-ions, usually simple anions or cations, such as chloride or sodium ions.

2. *Sample application and adsorption*, in which solute molecules carrying the appropriate charge displace counter-ions and bind reversibly to the gel. Unbound substances can be washed out from the exchanger bed using starting buffer.

3. *Start of desorption*, substances are removed from the column by changing to elution conditions unfavorable for ionic bonding of the solute molecules. This normally involves increasing the ionic strength of the eluting buffer or changing its pH. The molecules are released from the column in order of their binding strengths; the most weakly bound substances are eluted first.

4. *End of desorption*, substances not eluted under the previous experimental conditions are removed.

5. *Regeneration*, re-equilibration of the column at the starting conditions for the next purification.

In IEC one can choose whether to bind the substances of interest and allow contaminants to pass through the column, or to bind the contaminants and allow the substances of interest to pass through. Generally, the first method is more useful since it allows a greater degree of fractionation and concentrates the substances of interest.

The matrix of an ion exchanger consists in charged groups that have been covalently bound. Most ion exchangers are either anion exchanger or cation exchanger. An anion exchanger is positively charged and binds anions. Typical anion exchange groups are: diethylaminoethyl (DEAE) and quaternary ammonium (Q). Similarly, cation exchangers carry negatively charged groups and bind cations. Typical cation exchange groups are: carboxymethyl (CM) and methyl sulphonate (S). Q and S ion exchangers are known as strong ion exchangers, because they have a extreme pK_a values and are charged at a wide range of pH; while DEAE and CM are known as weak ion exchangers because they are weakly dissociated, and therefore uncharged, they act in a narrow pH range (Janson and Rydén,1998).

The capacity of an ion exchanger is a quantitative measure of its ability to take up exchangeable counter-ions and is therefore of major importance. It can be expressed as

total ionic capacity, available capacity or dynamic capacity. The total ionic capacity is the number of charged substitute groups per gram dry ion exchanger or per ml swollen gel; it is measured by titration with a strong acid or base. The actual amount of protein which can be bound to an ion exchanger, under defined experimental conditions; this is the available capacity for the gel. If the defined conditions include the flow rate at which the gel was operated, the amount bound is referred to as the dynamic capacity for the ion exchanger (AmershamBiosciences,2002).

3.3.4.5 Hydrophobic Interaction Chromatography

Hydrophobic interaction chromatography (HIC) is a popular technique for purifying proteins because it displays binding characteristics complementary to other protein chromatography techniques such as IEC.

HIC is a versatile technique that exploits the often small differences in surface-located hydrophobic patches of the proteins. This method is more effective at an early stage of a purification strategy and also for concentration. It generally functions by the principle of group separation and normally more than 50% of the extraneous impurities are removed, including the major portion of the proteolytic enzymes. HIC offers relatively high adsorption capacity combined with good selectivity and satisfactory yield of active material (Janson and Rydén,1998).

In HIC proteins are separated based on differences in their content of hydrophobic amino acid side chains on their surface. The separation takes place by differential interaction with individual alkyl or aryl substituents on neutral, hydrophilic carriers such as agarose gels. The binding to the HIC adsorbent is primarily driven by the weak hydrophobic interaction within the aqueous solvent and to a lesser extent by the creation of Van der Waals interactions. The strength of the binding depends on the density of hydrophobic groups on the surface, or near the surface of the protein and on the type and degree of substitution of the hydrophobic ligand coupled to the polymer matrix (Scopes,1987).

HIC requires the presence of moderately high concentration salt (1-2 M). The adsorbed proteins are typically eluted differentially either by decreasing the salt concentration and or by increasing the eluent concentration of polarity perturbants such as ethylene glycol.

The most widely used ligands for HIC are straight chain alkanes ($-C_n$) or simple aromatic compounds, like methyl, ethyl, propyl, butyl, phenyl, pentyl, hexyl, heptyl, octyl.

Temperature is an important factor to control in HIC, increasing the temperature enhances hydrophobic interactions; conversely, decreasing the temperature decreases hydrophobic interactions. Lowering the temperature generally causes a protein to be eluted earlier, and as a sharper peak.

3.3.4.6 Affinity Chromatography

Affinity chromatography (AC) separates proteins on the basis of a reversible interaction between a protein (or group of proteins) and a specific ligand coupled to a chromatographic matrix. The technique offers high selectivity, hence high resolution, and usually high capacity for the protein of interest (AmershamBiosciences,2002).

Affinity methods are based on biological pair formations of the type listed in Table 5.
The term of affinity chromatography is also used for separation methods based on more or less specific interactions between proteins and a variety of ligand molecules covalently attached to a solid phase.

Table 5. Examples of biospecific pairs utilized in classical affinity chromatography

Enzyme	Substrate, substrate analog, cofactor, cofactor analog, inhibitor
Hormone	Carrier protein, receptor
Antibody	Antigen
Glycoprotein	Lectin
Nucleic acid	Complementary polynucleotide, Polynucleotide-binding protein
Metal ions	Poly (His) fusion proteins, native proteins with histidine, cysteine and/or tryptophan residues on their surfaces

Source: (Janson,2006)

Classical biospecific affinity chromatography is primarily based on the use of immobilized enzyme inhibitors such as substrate analogs or cofactor analogs. Other methods are also very used based on the formation of chelated metal-ion complexes and charge transfer complexes (AmershamBiosciences,2002).

Immobilized metal affinity chromatography (IMAC) offers a selectivity factor apparently mainly dependent on the number of available histidine residues on the surface of a protein. In the synthesis of an IMAC adsorbent, a metal chelate complex forming group such as iminodiacetic acid is covalently attached to a suitable support such as bead-shaped agarose. Before use, the chelating groups are saturated with the appropriate metal ion. The most useful are Cu^{2+} and Zn^{2+}, but Co^{2+} and Ni^{2+} are also used.
Other important affinity purification methods are the ones that utilize ligands like lectins for glycoprotein purification, immuno ligands such as monoclonal antibodies for isolation of a variety of proteins (Janson and Rydén,1998).

In any design of an affinity separation experiment, it is important to consider, in addition to the choice of ligand, the choice of support and chemistry for the immobilization of the ligand (AmershamBiosciences,2002).

3.3.5 Electrophoresis

Electrophoresis in gels (primarily polyacrylamide gels), is a universally applied technique for protein analysis. Electrophoresis has developed during the last decades, and innovative equipments and different gels media can be found and is considered a practical analytical tool in a protein laboratory (PharmaciaBiotech,1995).

Electrophoresis consists in the migration of all kinds of charged particles in an electrical field. The separation is performed in a supporting gel medium. The gel can either act as an inert support for the electrophoresis buffer or actively participate in the separation by interacting with the proteins. In the latter case, the protein-gel interaction is the actual separation factor, whereas the electrical field merely makes the proteins migrate through the gel (Janson and Rydén,1998).

This is the method of choice for gathering information about the composition of a crude sample. It gives an approximation of the molecular weight and isoelectric point, together with a distribution profile of MW and pI of contaminating proteins. It also gives information about the purity of a protein after a purification step.

Sodium dodecyl sulfate electrophoresis (SDS-PAGE) is the method used to determine the MW of a protein, using markers of high and low molecular weight. The protein solution to be analyzed is often treated by boiling for a short period (5 minutes) with reducing agents like β-mercaptoethanol. Native electrophoresis (native-PAGE) is the method used to study the composition and structure of native proteins. Since both the conformation and biological activity of proteins remain intact during this technique, it determines the purity of a protein, without denaturing it (PharmaciaBiotech,1995).

Isoelectric focusing (IEF) is a technique which separates proteins according to differences in their isoelectric points. It can also be applied to determine the pI of a pure protein.
Two dimensional electrophoresis combining IEF and SDS-PAGE is one of the most powerful methods available for analyzing complex protein mixtures.
Electrophoresis has a number of practical advantages, including (Janson and Rydén,1998):

- Relatively simple and inexpensive equipment.
- High resolution results.
- Ease of multiple sample analysis.
- High sensitivity.
- Specific detection easy.
- Aesthetic appearance of banding pattern.

The separation pattern is visualized in the gel using some protein staining procedure. The most frequently used stain is Coomassie Brillant Blue (CBB.) The proteins are first precipitated by agents such as trichloroacetic acid and/or formaldehyde. The gel is then exposed to a solution of stain dissolved in a dilute acetic acid/ethanol mixture. Finally, excess stain is removed by soaking the gel in a distaining solution of acetic/acid/ethanol until the background is clear (PharmaciaBiotech,1995).

The most sensitive protein-staining method is based on the deposition of silver, which are often claimed to be up to 100 times as sensitive as conventional CBB-based methods, with detection limits of less than 1 ng/mm^2 of protein (Janson and Rydén,1998). The staining strategy consists in fixing and removal of buffer ions, "sensitizing" the proteins in a glutardialdehyde solution, removal of excess glutardialdehyde, and reaction with silver ions in a silver nitrate solution and stopping the development in acetic acid. A final rinsing step in 10 % acetic acid / 5 % glycerol is used to prevent gradient gels from curling or cracking after drying (PharmaciaBiotech,1995).

3.4 CHARACTERIZATION OF ENZYMES

After following the established protocol for the purification of the protein the purified enzyme is obtained, and the biochemical properties have to be determined like pH optimum and temperature optimum, isoelectric point, molecular weight, specificity for the substrate, amino acid composition.

3.4.1 pH Optimum

Enzymes are active over a limited range of pH values. The range may be narrow or broad. The optimum pH of an enzyme may differ for different substrates and substrate concentration, it can change depending on the enzyme source and the enzyme structure, stability of the enzyme, temperature and length of the reaction period (Reed,1975).

The pH optimum is the pH value, at which the enzyme activity is the highest. When the pH is changed from of the optimum the enzyme activity decreases.

The initial rates for many enzymatic reactions exhibit bell-shaped curves as a function of pH and are called pH-activity curves. These curves reflect the ionizations of certain amino acid residues that must be in a specific ionization state for enzyme activity (Voet and Voet,1990). Figure 5 shows the pH-activity curve of an enzyme.

3.4.2 Temperature Optimum

The rate of the enzymatic reactions generally increases with temperature within the range of temperature at which the enzyme is active and stable. The rate doubles its value per each 10ºC in temperature increased.

Enzymes have an optimum temperature value, at which its catalytic activity is the maximum; if the temperature exceeds the optimum value the enzyme can be inactivated or denatured.

At very low temperatures, the rate of enzymatic reactions is very slow or it does not occur, but the catalytic action appears when the temperature has a normal value, at higher temperatures the rate of enzymatic reactions increase, but the rate of the inactivation of the enzyme also increases. These two factors have opposite effects on the rate of the enzymatic reaction. At low temperatures, the rate of enzyme inactivation is so slow that it does not have to be considered. At extremely high temperatures, inactivation is almost immediate and little or no transformation of the substrate can take place. At intermediate temperatures the extent of hydrolysis depends on both factors (Reed,1975).

Temperature-activity curves have the well-known shape shown in Figure 5. The highest point in the curve is the optimum value, far from this value the enzymatic activity decreases.

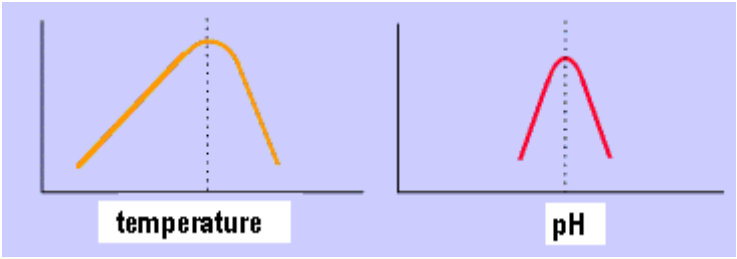

Figure 5. Temperature and pH activity curves

3.4.3 Isoelectric point

The isoelectric point (pI) is the pH at which a molecule carries no net electrical charge; this means that the positive charges and the negative charges are equal. In order to have a sharp isoelectric point, a molecule must be amphoteric, meaning it must have both acidic and basic functional groups. Proteins and amino acids are common molecules that meet this requirement (Nelson,2004).

Proteins can be separated according to their isoelectric point in a process known as isoelectric focusing.

At a pH below the pI, proteins carry a net positive charge. Above the pI they carry a net negative charge. This has implications for running electrophoretic gels. The pH of an electrophoretic gel is determined by the buffer used for that gel. If the pH of the buffer is above the pI of the protein being run, the protein will migrate to the positive pole (negative charge is attracted to a positive pole). If the pH of the buffer is below the pI of the protein being run, the protein will migrate to the negative pole of the gel (positive charge is attracted to the negative pole). If the protein is run with a buffer pH that is equal to the pI, it will not migrate at all. This is also true for individual amino acids (Nelson,2004).

3.4.4 Molecular weight

The molecular weight is a highly specific characteristic of a molecule and is often the first physicochemical property that is measured of a protein. Traditionally, SDS-PAGE, analytical ultracentrifugation or gel filtration chromatography are used for molecular weight determinations. Recently, mass spectrometric techniques have been developed that permit mass determination of intact proteins (Jensen, Shevchenko et al.,2002).

3.4.5 Specificity for the substrate

One of the properties of enzymes that makes them so important as diagnostic and research tools is the specificity they exhibit relative to the reactions they catalyze. A few enzymes exhibit absolute specificity; that is, they will catalyze only one particular reaction. Other enzymes will be specific for a particular type of chemical bond or functional group (Stryer, Berg et al.,2002).

In general, there are four distinct types of specificity:

- Absolute specificity - the enzyme will catalyze only one reaction.
- Group specificity - the enzyme will act only on molecules that have specific functional groups, such as amino, phosphate and methyl groups.
- Linkage specificity - the enzyme will act on a particular type of chemical bond regardless of the rest of the molecular structure.
- Stereochemical specificity - the enzyme will act on a particular steric or optical isomer.

3.4.6 Amino acid composition

Amino acid analysis is one of the best quantitative methods in protein chemistry and used by many laboratories for peptide/protein characterization.

For quantitative determination of amino acids in a protein the first step is the hydrolysis of the sample with 6 N HCl containing a small amount of phenol. The protein peptide bonds are hydrolyzed by the HCl vapors under vacuum. The hydrolysis is carried out during 24 and/or 72 hours at 110ºC in evacuated and sealed tubes. The 72 hours is needed to determine valine and isoleucine composition which only have 60% recovery in the 24 hours run, but 98% recovery in 72 hours.

Cysteine, methionine and tryptophan are destroyed during the hydrolysis with 6N HCl. Cysteine and methionine can be determined by oxidation with performic acid, yielding the acid stable forms cysteic acid and methionine sulfone, prior to the standard aicd hydrolysis. Tryptophan can be quantified but requires another test with 3M mercaptoethane sulfonic acid (MESA) (Eaker,2006).

During the hydrolysis glutamine and aspargine will turn into glutamic acid and aspartic acid. To determine these amino acids the ammonia concentration can be used.

After the hydrolysis the determination of the amino acids can be done following a separation before or after derivatization with a group-specific reagent. Separation of free amino acids can be done by ion exchange chromatography followed by post-column detection with nynhidrin, OPA (ortho-phthalaldehyde/thiol) or fluorescamine (Eaker,2006).

3.4.7 Enzyme kinetics

Living systems depend on chemical reactions which, on their own, would occur at extremely slow rates. Enzymes are catalysts which reduce the needed activation energy so these reactions proceed at rates that are useful to the cell.

In most cases, an enzyme converts one chemical known as *substrate*, into another known as *product*. Figure 6 represents a graph of product concentration versus time that follows three phases. At very early time points, the rate of product accumulation increases over time (1). For an extended period of time, the product concentration increases linearly with time (2). At later times, the substrate is depleted, so the curve starts to level off (3) (Nelson,2004).

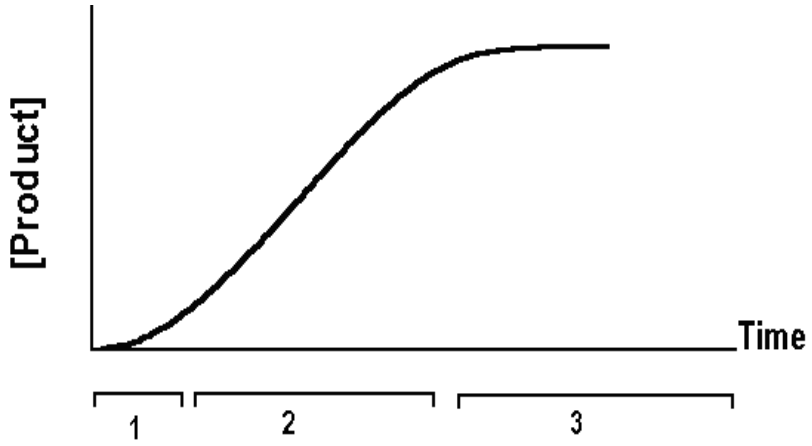

Figure 6. Product concentration during an enzyme reaction

Enzyme kinetics fit the initial velocity of the enzyme reaction as a function of substrate concentration. The velocity of the enzyme reaction is the slope of the linear phase, expressed as amount of product formed per time.

The enzyme velocity is measured at different substrate concentrations and the graph is represented in Figure 7 (White, Handler et al.,1983).

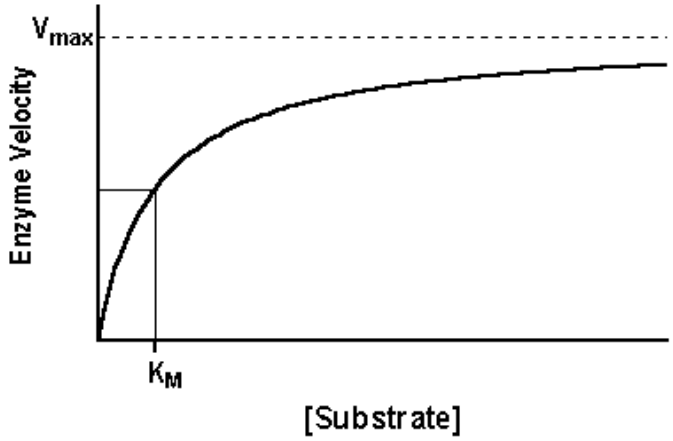

Figure 7. Enzyme velocity as a function of substrate concentration

Enzyme velocity as a function of substrate concentration often follows the Michaelis-Menten equation (Ec 1):

$$\text{Velocity} = V = \frac{V_{max}[S]}{[S] + K_M} \qquad \text{Ec 1}$$

Where Vmax is the limiting velocity as substrate concentrations get very large. Vmax (and V) are expressed in units of product formed per time. If the molar concentration of enzyme is known, the observed velocity can be divided by the concentration of enzyme sites in the assay, and express Vmax as units of moles of product formed per second per mole of enzyme sites. This is the *turnover number*, the number of molecules of substrate converted to product by one enzyme site per second. In defining enzyme concentration, distinguish the concentration of enzyme molecules and concentration of enzyme sites (if the enzyme is a dimer with two active sites, the molar concentration of sites is twice the molar concentration of enzyme).

K_M is expressed in units of concentration, usually in Molar units. K_M is the concentration of substrate that leads to half-maximal velocity (Voet and Voet,1990).

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SUMMARY OF THE WORK

The aim of the experimental work was to study the glycosidases in babaco peel, pulp and latex. The glycosidic activities were determined also in papaya latex in order to make a comparison with babaco latex. The α-mannosidase in freeze-dried babaco latex was purified and characterized.

The first part of this work was a screening for glycosidases in different parts of babaco. The tested enzymes were: α-arabinosidase, β-glucosidase, β-fucosidase, β-galactosidase, acetyl β-glucosaminidase and α-mannosidase. An efficient method for the glycosidases extraction by means of pH and buffer concentration was developed and the final conditions found for an optimum extraction were 30 mg/ml of freeze-dried sample (peel, pulp and latex) in 20 mM Tris pH 7.5. A method for obtaining the optimum glycosidic activities was developed, 50ºC and 30 minutes of incubation, with a substrate concentration of 5 mM in 200 mM of sodium acetate pH 4.5, gave the maximum activity of the enzymes. The results are presented in *Paper I*. The α-mannosidase was the glycosidic enzyme that had the highest activity mainly in freeze-dried babaco latex. The activities of the other glycosidases were significantly lower.

The second part of this study consisted in the purification and characterization of an α-mannosidase of the freeze-dried babaco latex. For the isolation of the enzyme different chromatographic steps were used: ion-exchange chromatography (Q-Sepharose), affinity chromatography (Con A Sepharose) and gel filtration (Superdex 200). The final purification protocol of α-mannosidase is presented in Figure 6. The enzyme was characterized by means of optimum temperature and pH, kinetics, molecular weight, isoelectric point, effects of inhibitors and activators, stability and amino acid composition. The results are presented in *Paper II*.

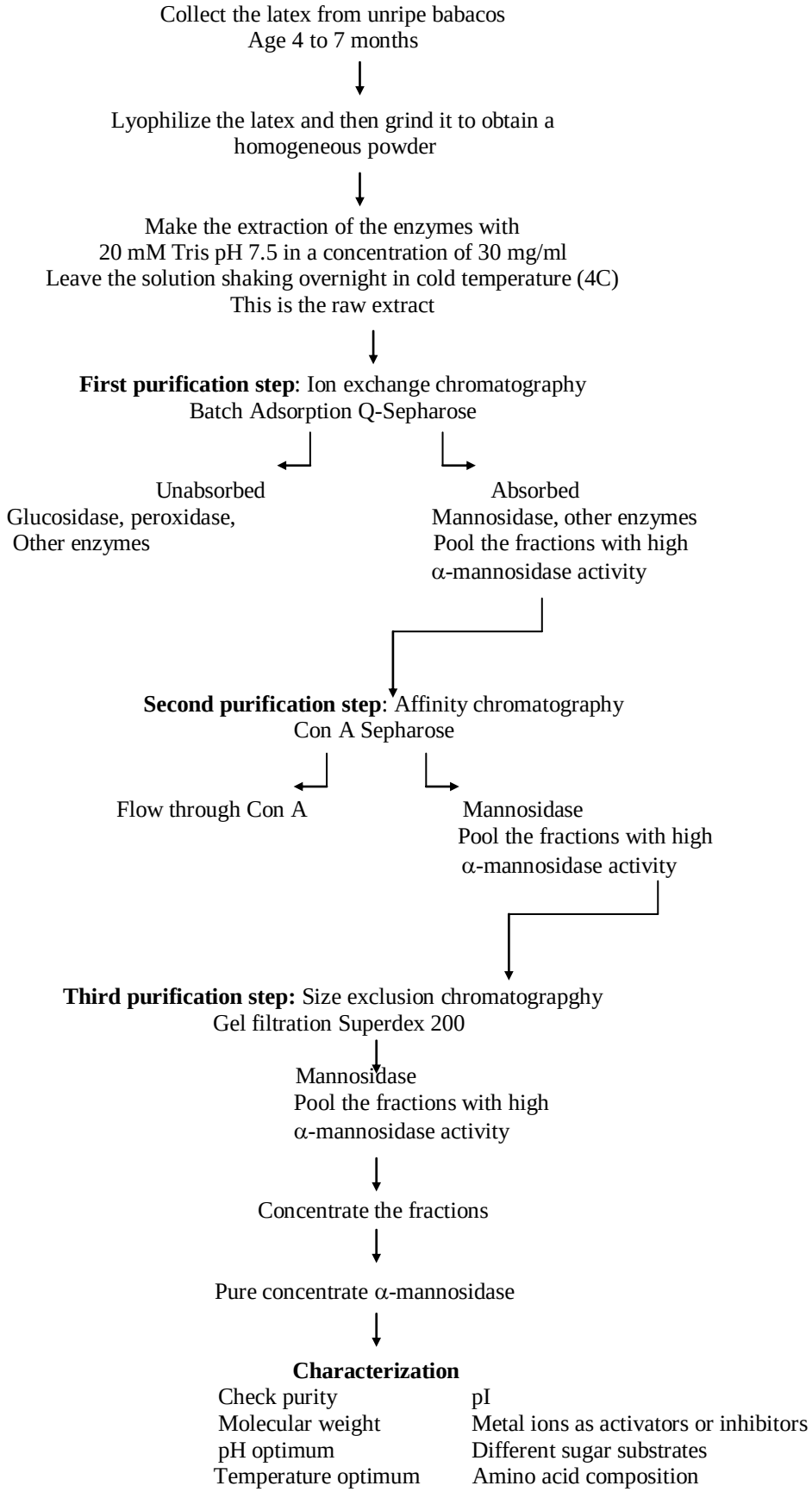

Figure 8. α-Mannosidase purification protocol

PAPER I

A screening of glycosidases in babaco
(*Vasconcellea x* Heilbornii cv. babaco)

A screening of glycosidases in babaco (*Vasconcellea x* Heilbornii cv. babaco)

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Key words: babaco latex / glycosidases / papaya latex / sugar enzyme / Vasconcellea x Heibornii cv. babaco

Abstract

The objective of this research project is to investigate the babaco fruit for enzymes, mainly sugar degrading enzymes, glycosidases. An initial screening for glycosidases in different parts of babaco was made. The tested enzymes were: α-arabinosidase (EC 3.2.1.55), β-glucosidase (EC 3.2.1.21), β-fucosidase (EC 3.2.1.38), β-galactosidase (EC 3.2.1.23), acetyl β-glucosaminidase (EC 3.2.1.14) and α-mannosidase (EC 3.2.1.24).

The main aim has been to develope a method for the glycosidase extraction in peel, pulp and latex from babaco fruit; and to optimize the reaction conditions for the glycosidic activity assay. The final conditions for the extraction were 30 mg/ml of freeze-dried sample (peel, pulp or latex) in 20 mM Tris pH 7.5. An effective method to evaluate the activity of glycosidases consists in an incubation of enzyme and substrate at 50 ºC for 30 minutes; the substrate concentration is 5 mM in 200 mM of sodium acetate pH 4.5.

The α-mannosidase presents the highest activity in babaco latex. The activities of the other glycosidases in peel, pulp and latex of babaco were significant lower than that of α-mannosidase. The glycosidase activities of papaya latex were determined in order to compare babaco latex with papaya latex, and it was shown that the activity of α-mannosidase in babaco latex is higher than in papaya latex, although the latex of papaya presents a significant activity of α-mannosidase and β-glucosaminidase.

Introduction

Babaco (*Vasconcellea* x Heilbornii cv. babaco) is a native fruit from Ecuador that has excellent sensorial properties and is a good enzymatic source. It is rich in different enzymes mainly proteases and lipases, but also peroxidases and glycosidases (Villarreal, Dhuique-Mayer et al.,2003).When babaco fruit is totally green exudates a white milky substance called latex that is comparable with papaya latex (*Carica papaya L.)* a close relative to babaco. It has previously been shown that significant β-glucosidase activity exists in papaya fruit (Hartmann-Schreier and Schreier,1986).

A group of sugar degrading enzymes are known as glycosidases. Their primary role in plants and animals is to hydrolyze glycosides. Each type of glycosidase is probably a group of enzymes that hydrolyze the same type of bond, but have different specificity. The affinity of the enzyme for a particular substrate is dependent upon the nature of the enzyme source (Woodward and Wiseman,1982).

Glycosidases are known to participate in fruit ripening and plant defence. They are also very important in food industry because they enhance the flavor of some products because of the polysaccharide degradation. They are used in brewery and bakery (Kirk and Othmer,1963; Waln and Poulton,1987; Asano,2003; Sorensen, Kragh et al.,2004)

Materials and Methods

Sample preparation

The babaco fruits for these studies were obtained from a green house plantation, located 70 km toward the North valley of Quito, Ecuador. Green unripe fruits were used for the analyses because it is shown that ripe fruits present low enzymatic activity (Villarreal, Dhuique-Mayer et al.,2003).The peel was separated from the pulp and both preparations were frozen at -20 ºC.

The babaco latex was collected from unripe fruits that were between 4 to 7 months. A superficial cut was made along 3 of the 5 faces of the fruit with a stainless steel blade,

and the latex was allowed to drop into a sterilized recipient. The fruits were left on the tree until they were ripe for harvest (Madrigal, Ortiz et al.,1980).

All the samples were cooled, frozen at -20 ºC and lyophilized (Lyovac GT2). They were ground and stored in plastic containers at 0 ºC until the enzymatic analyses were performed.
For the papaya latex the same procedure of extraction, drying and storage was followed.

Extraction of glycosidases

A method was developed for the extraction of glycosidases from freeze-dried babaco peel, pulp and latex. The development of the method included evaluation of mechanical extraction conditions, buffer ionic strength and sample concentrations. Three consecutive extractions were made with Tris buffer of different salt concentrations 20 mM Tris; 20 mM Tris / 100 mM NaCl; 20 mM Tris / 200 mM NaCl; 20 mM Tris / 500 mM NaCl.

The suspensions were shaken at room temperature for 2 hours and then in a cold room overnight. The samples were centrifuged at 11000 g for 15 minutes. The supernatant was filtered through a 0.22 µm filter and stored at 4 °C until use. The obtained solution is referred to as "raw extract". Generally, the raw extract should be used within 2 weeks to avoid bacterial growth. The protein concentration and the enzyme activity of the different glycosidases were analyzed in all the extractions.

Different concentrations of babaco latex were tried: 15, 30 and 60 mg/ml in order to determine the optimum concentration of the sample to obtain a high glycosidic activity.

Optimization of pH and temperature for the glycosidic activity

The sample was incubated for one hour at room temperature (22ºC), at 37°C and 50°C; different buffer pHs were tried: 4, 4.25, 4.5, 4.75, and 5 based on the studies of Hartmann and Schreier (1986) about the β-glucosidase from papaya fruit.

Determination of protein content

The protein concentration was analyzed following the micro titer plate protocols for the Bradford method using the Bradford Reagent for protein assay from SIGMA. A standard curve for concentration determination was made with BSA as a representative protein. The linear range of this micro titer plate assay is from 0 μg/ml to 10 μg/ml.

It is however important to be aware of the fact that different proteins will give different values for the protein concentration in the test, depending on the nature of the protein and its reaction with the dye.

Into separate micro titer plate wells 150 μl of each standard or sample were pipetted together with 150 μl of Bradford dye reagent. The sample and reagent were mixed thoroughly for at least 10 minutes using a micro plate mixer at room temperature as the absorbance increases over time. Absorbance was measured at 595 nm using a Labsystem Multiskan MS microtiter plate spectrophotometer.

Samples and standards were analyzed in triplicates. The solutions to be determined have to be diluted in order to be within the linear range of the micro titer plate assay.

Determination of glycosidase activity

In order to identify the different glycosidase activities in the babaco, synthetic substrates were used for the different enzymes. The substrates tested are presented in **Table I** with the enzymes and the respective EC numbers.

Table I. List of substrates tested in babaco fruit

Substrate	***Enzyme***	***EC number***
p-nitrophenyl α-L-arabinopyranoside (pNPA)	α-arabinosidase	EC 3.2.1.55
p-nitrophenyl-β-D-glucopyranoside (pNPG)	β-glucosidase	EC 3.2.1.21
p-nitrophenyl β-D-fucopyranoside (pNPF)	β-fucosidase	EC 3.2.1.38
o-nitrophenyl β-D-galactopyranoside (pNPGal)	β-galactosidase	EC 3.2.1.23
p-nitrophenyl N-acetyl β-D-glucosaminide (pNPAG)	Acetyl -β-glucosaminidase	EC 3.2.1.14
p-nitrophenyl α-D-mannopyranoside (pNPM)	α-mannosidase	EC 3.2.1.24

Substrate solutions containing 5 mM substrate in 200 mM Na Acetate pH 4.5 were used for each of the enzymes (Hartmann-Schreier and Schreier,1986).

In each micro titer plate well 10 µl of sample was mixed with 100 µl of the reaction mixture. The plates were shortly shaken gently and then incubated for 30 minutes at 50°C. In the presence of active enzyme the product p-nitrophenol is released from the substrates. P-nitrophenol is a yellow dye substance that can be monitored at 405 nm. After the incubation period 100 µl of 1M sodium carbonate was added in order to stop the reaction and increase the color for improved detection. The absorbance was measured at 405 nm in a micro plate spectrophotometer Labsystem Multiskan MS.

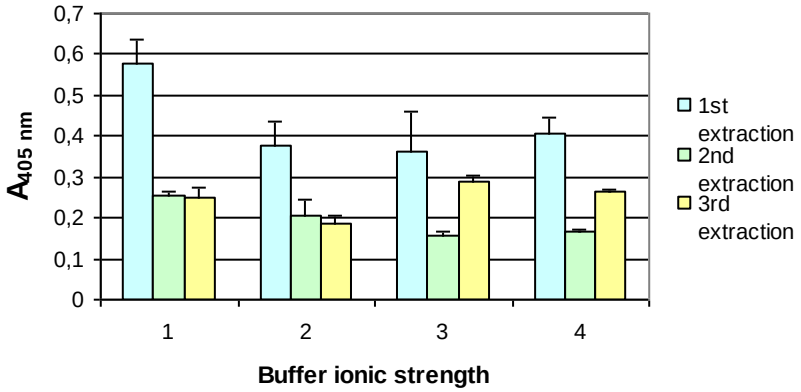
Samples were analyzed in triplicates, including subtraction of the absorbance readings from substrate background control and sample background controls.

Sometimes samples had to be diluted in order to obtain absorbance values within the linear range of the micro titer plate assay. At dilutions over 100 times a solution that contains 0.25% of BSA with 20 mM Tris is used to dilute the sample, to avoid adsorption of the enzyme in tubes. In adittion albumin protects the activity of the enzyme.

A unit of glycosidic activity was defined as the amount of enzyme that hydrolyzes 1 µmol of p-nitrophenol ($\varepsilon_{450} = 18.5 \times 10^3\ M^{-1}\ cm^{-1}$) per minute at pH 4.5 and 50ºC. The activity was expressed by Units of activity per gram of dried sample (Hartmann-Schreier and Schreier,1987).

Results and Discussion

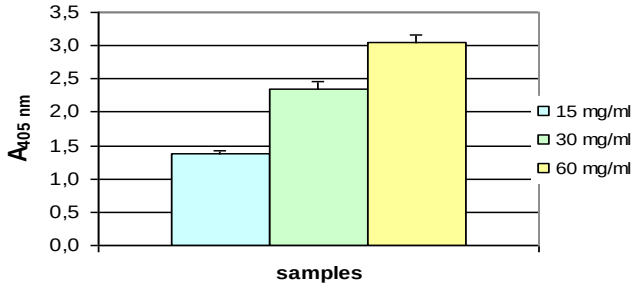
The optimum extraction of the glycosidases from babaco was done with 20 mM buffer Tris pH 7.5 without salt. This buffer gave the highest glycosidic activity compared with the extractions that were made with Tris buffer containing salt. Shaking the samples all night in a cold room is necessary to obtain most of the glycosidases in only one extraction. The results about the dependence of buffer ionic strength for β-glucosidase from babaco latex are presented in **Figure 1.**



Three continue extractions were made with Tris buffer with different salt concentrations **1)** 20 mM Tris **2)** 20 mM Tris / 100 mM NaCl **3)** 20 mM Tris / 200 mM NaCl **4)** 20 mM Tris / 500 mM NaCl.
The β-glucosidase activity was determined under the standard conditions (60 min; 50ºC)

Figure 1. Dependence of buffer ionic strength for β-glucosidase extractions from babaco latex

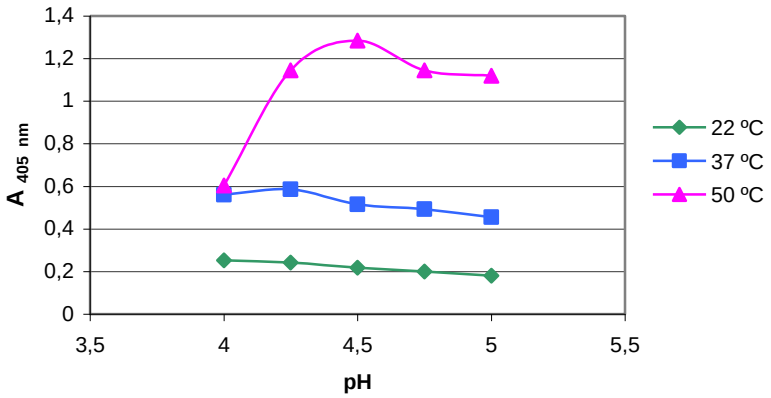
When different amounts of latex in the extract mixtures were tried it was shown that higher latex concentrations did not increase glycosidase activity in proportion to the increase of concentration. It seems that the solution was saturated with the latex compounds. The glycosidase activity was sufficiently high at 30 mg/ml and did not increase proportionally at higher concentrations. A concentration of 30mg/ml was therefore selected as standard concentration in the extraction procedure to avoid unnecessary waste of latex (**Figure 2**).



Different sample concentrations: 15, 30 and 60 mg/ml . The β-glucosidase activity was determined under the standard conditions (60 min; 50ºC)

Figure 2. Different concentrations of babaco latex in β-glucosidase activity

The pH and temperature optimum for the glycosidase activity were determined and the enzyme β-glucosidase from babaco latex was used as reference. In the case of babaco the highest activity of the glycosidases was obtained at pH 4.5 and 50°C for 30 minutes of incubation, at these conditions the enzyme showed good stability. The results are presented in the following **Figure 3**.



Incubation was done for one hour at 22 ºC, 37 °C and 50 °C. Different buffer pHs were tried: 4, 4.25, 4.5, 4.75, and 5.

Figure 3.Temperature and pH dependence of β-glucosidase activity in babaco latex

These results can be compared with the ones obtained by Hartmann (1987) in *carica papaya* for the β-glucosidase, the optimum pH and temperature for enzyme activity were at 5.0 and 50ºC, respectively.

In comparison with peel and pulp the latex of the babaco has the highest protein content (**Table II**).

Table II. Protein concentration in babaco

Sample	**Protein Content** [1] **mg protein / g sample**
Babaco Peel	4.08 ± 0.93
Babaco Pulp	1.43 ± 0.41
Babaco Latex	285.65 ± 22.65

[1] mean ± standard deviation of triplicate samples

Some sugar degrading enzymes were identified in babaco and the glycosidic activities for each one were determined. The *a*-mannosidase presents the highest activity in babaco freeze-dried latex, followed by β-galactosidase in the babaco dried peel. The other glycosidases activities were significantly lower. The results are presented in **Figure 4**. Comparing with *carica* papaya among the glycosidases present in the fruit according to Hartmann (1986) β-galactosidase together with α-mannosidase and β-glucosidase showed the highest activity in extracts of the fruit dried pulp.

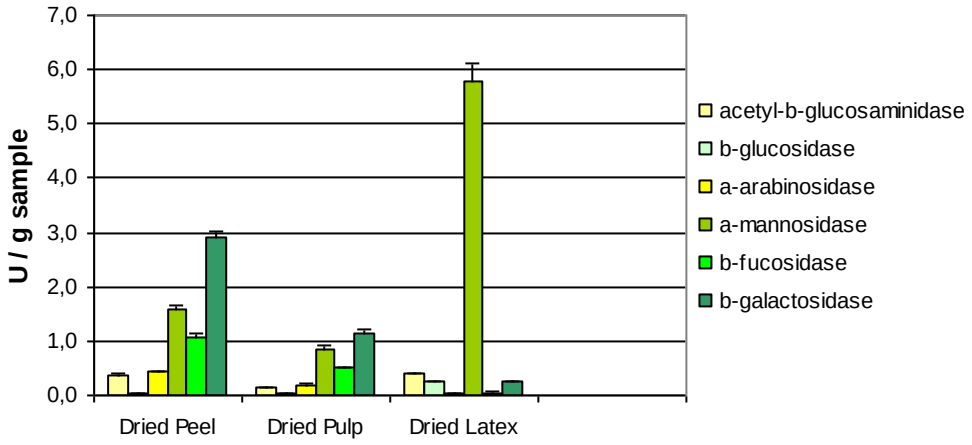


Figure 4. Glycosidases in babaco

The activity of each glycosidic enzyme was determined in the freeze-dried babaco latex and in the freeze-dried papaya latex. The babaco latex showed a higher α-mannosidase activity than papaya latex while papaya latex presents a highest acetyl β-glucosaminidase activity (**Figure 5**).

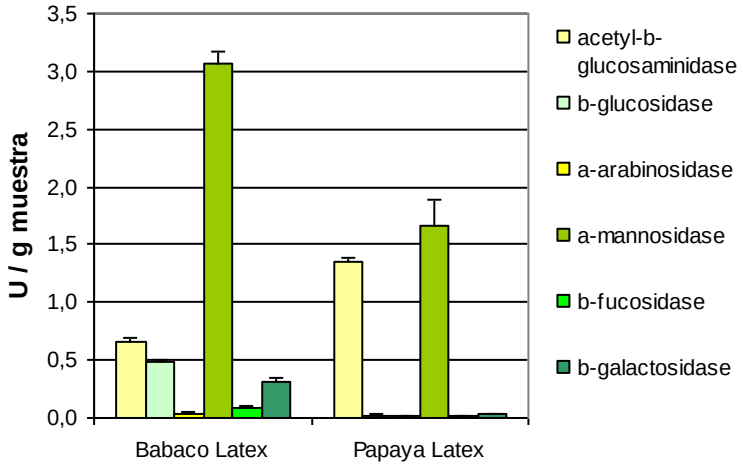


Figure 5. Glycosidases in babaco latex and papaya latex

Conclusions

- Glycosidases are used in food industry and babaco might to be a new source of interesting enzymes especially for α-mannosidase.
- The optimal conditions for the extraction of glycosidases were 30 mg/ml of freeze-dried sample in 20 mM Tris pH 7.5 and developed the suitable method for the enzymatic assay that consisted in an incubation of enzyme and substrate at 50 ºC for 30 minutes; the concentration of the substrate was 5 mM in 200 mM of sodium acetate pH 4.5.
- The α-mannosidase was the glycosidic enzyme with the highest activity in freeze-dried babaco latex; some studies about its purification and characterization are required to complement the research to find the optimal conditions at which this enzyme works.

Acknowledgements

This work had been done at the Center for Surface Biotechnology, in the Biomedical Centrum of the Uppsala University, Uppsala - Sweden, and at the Department of Food Science and Biotechnology of Escuela Politécnica Nacional, Quito – Ecuador, under the support of the International Program in the Chemical Sciences (IPICS) from Sweden

and the Program of Modernization of Agricultural Services (PROMSA) AQ-CV 010 project, from Ecuador. We gratefully acknowledge their financial assistance.

Abbreviations

pNPA, p-nitrophenyl α-L-arabinopyranoside; pNPG, p-nitrophenyl-β-D-glucopyranoside; pNPF; p-nitrophenyl β-D-fucopyranoside; pNPGal, o-nitrophenyl β-D-galactopyranodide; pNPAG, p-nitrophenyl N-acetyl β-D-glucosaminide; pNPM, p-nitrophenyl α-D-mannopyranoside; BSA, bovine serum albumin.

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PAPER II

Purification and characterization of an α-mannosidase from the tropical fruit babaco

***(Vasconcellea* x *Heilbornii* cv. babaco)**

Purification and characterization of an alpha-mannosidase from the tropical fruit babaco (Vasconcellea x Heilbornii cv. babaco)

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Keywords: α-mannosidase/ babaco/ latex/ purification/ characterization/ glycosidase

Abstract

An α-mannosidase (EC 3.2.1.24) present in the lyophilized latex of babaco (*Vasconcellea heilbornii* babaco) has been purified to apparent homogeneity by native PAGE. The purification involves a three step procedure with successive anion exchange with Q Sepharose, lectin affinity chromatography using ConA Sepharose and gel filtration using Superdex 200 prep grade. The molecular weight has been determined to be 280 kDa by Superdex 200 prep grade gel filtration. Isoelectric foucusing showed a pI range between 5.85 and 6.55, likely originating from occurrence of slighty different glycol isoforms. The optimal temperature for the α–mannosidase was determined to lie between 50 ºC and 60ºC, and the optimal pH was 4.5 at 50 ºC. The K_M value for p-nitrophenyl alpha mannopyranoside (pNPM) was found to be 1.25 mM and the V_{max} was 2.4 μkat·mg^{-1} at 50ºC and 1.94 μkat·mg^{-1} at 40ºC The pure α–mannosidase was specific for mannose and did not show any activity for other tested synthetic substrates.

Introduction

Babaco (*Vasconcellea pentagona* Heilbornii) is a fruit native from Ecuador and is widely appreciated for its fresh taste and pleasant aroma. It has earlier been shown that the latex of green fruits of papaya, a close relative to babaco and famous for its high content of proteases (ElMoussaoui, Nijs et al.,2001; Azarkan and ElMoussaoui,2003) also contains significant levels of some glycosidases, notably β-glucosisdase,

β-galactosidase and α-mannosidase (Hartmann-Schreier and Schreier,1986). Babaco and its closest relatives have recently been rehabilitated into their own genus and are no longer considered to be a section within the genus of *Carica* (Kim and P.H.Moore,2002; VanDroogenbroeck and Breyne,2002; VanDroogenbroeck and Kyndt,2004). The close relationship and the similarities between the two genuses in terms of the enzymes studied so far can provide useful information with regards to enzyme content and characteristics. A recent example of this can be seen in a study of the cysteine proteases present in babaco (Kyndt, VanDamme et al.,2007).

Sugar degrading enzymes, *glycosidases,* play key roles in many biological processes (Henrissat and Coutinho,2001; Coutinho and Stam,2003). Plant α-mannosidases are believed to, together with other glycosylases, take part in the degradation of polysaccharides, glycoproteins and glycoconjugates, both in seed germination and fruit development. There are not a large number of α-mannosidases isolated and characterized to date even though the *Canavalia ensiformis* (jack bean) α-mannosidase has been extensively studied (Li,1967; Snaith,1975; Einhoff, Wolfgang and Rüdiger,1988; Niyogi and Singh,1988). The identification of jack bean α-mannosidase as a Zinc metalloenzyme requiring Zn^{2+} for it full activity (Snaith,1975) has proven to be true also for other studied plant α-mannosidases. As part of our work in studying the enzymes of the babaco fruit we here describe the purification and characterization of a α-mannosidase present in the latex of the fruit.

Materials and methods

Latex collecting and Extractions

Lyophilized babaco latex was provided by Escuela Politecnica Nacional, Departamento de Ciencia de los Alimentos y Biotecnologia, Quito, Ecuador. The latex was collected from unripe fruits between 4-7 months old, originating from a green house plantation located in a valley North of Quito. A superficial cut was made along 3 of the 5 faces of the fruit with a stainless steel blade (Madrigal, Ortiz et al.,1980) and the drops of latex were collected in a sterilized container. Collected latex was lyophilized and frozen until use.

Extract was prepared shaking 13.5 g of lyophilized latex together with 450 ml of 20 mM Tris-HCl pH 7.5 (30 mg/ml) at room temperature for 1-2 hours prior to shaking in cold room for at least 8 hours. Particulate material was separated by centrifugation at 11000 g for 20 minutes followed by filtering of the supernatant through glass wool. Finally the extract solution was sterile filtered using a 0.2 µm filter (Sarstedt, Nümbrecht, Germany) and stored at 4°C until use.

Determination of the protein content

The protein content of extractions and the different purification steps including the final α-mannosidase, were determined by using the Bio-Rad Protein Microassay (Bio-Rad Sweden, Stockholm, Sweden) following the Microtiter Plate Protocol.

Activity Assays

All assays for glycosidase activity in extracts, fractions and pure α-mannosidase were performed in triplicates in the same manner unless otherwise stated, using p-nitrophenyl α-mannopyranoside (pNPMα) and various other nitrophenyl substrates (Sigma-Aldrich, Stockholm, Sweden). The standard assay was performed by incubation of 10 µl of sample with 100 µl of 5 mM substrate in 100 mM NaAc pH 4.5 for 30 minutes at 50°C in a microtiter plate well (Sarstedt, Landskrona, Sweden). The reaction was stopped by the addition of 100 µl of 1 M Na_2CO_3 and the absorbance was read at 405 nm using a Labsystem Multiskan MS microplate spectrophotometer (Thermo Labsystems, Stockholm, Sweden). Subtraction was made for background absorbance from substrate and sample. All dilutions of enzyme solutions were performed with 0.25 mg/ml BSA in 20 mM Tris-HCl pH 7.5.

Purification of the α-mannosidase

All chromatographic equipment, gels and standards were supplied by Amersham Biosciences, Uppsala, Sweden (now GE Healthcare). Chromatography was performed using a FPLC system at room temperature and absorbance monitoring at 280 nm. All fractions were stored at 4ºC after collection.

(a) Anion Exchange Chromatography. A volume of 450 ml of babaco latex extract (30 mg/ml latex in 20 mM Tris-HCl pH 7.5) was batch adsorbed to approximately 30 ml Q Sepharose HP gel previously equilibrated with 20 mM Tris-HCl pH 7.5. The

extract and gel suspension were incubated with shaking on a rotary shaker at 25 °C for 1 hour after which the gel was separated from unadsorbed material by suction filtering. The gel was washed carefully with approximately 5 gel volumes of 20 mM Tris-HCl pH 7.5 and was then packed in a XK26/20 column (2.6 x 20 cm). Additional washing with 100 ml of 20 mM Tris-HCl pH 7.5 was performed at 3 ml/min before applying a stepwise gradient of 1 M NaCl in the same Tris-HCl buffer. Two steps of 0.1 M NaCl for 115 ml and 0.2 M NaCl for 60 ml respectively were applied before elution of the α-mannosidase containing material by 90 ml of 0.3 M NaCl. Throughout the experiment 14 ml fractions were collected. Fractions with high α-mannosidase activity were pooled for further purification.

(b) Con A Affinity Chromatography. A volume of 36 ml from a total volume of 42 ml of pooled fractions from Q Sepharose HP chromatography was applied to a Con A Sepharose 4B column (1.0 × 5.0 cm) equilibrated with 20 mM Tris-HCl pH 7.5 containing 0.5 M NaCl at 0.5 ml/min. The column was washed until the absorbance value of the eluate reached baseline. The bound α-mannosidase was eluted with 0.5 M α- methyl-D-mannopyranoside in the same buffer at 0.1 ml/min. Directly at the start of increase of absorbance in the eluate at 280 nm the system was paused for 6 hours and the gel was incubated in the presence of α- methyl-D-mannopyranoside before elution was continued. All eluted material was collected in one fraction (fraction 1) until the absorbance at 280 nm was lower than 0.05. At this point the system was paused again for one hour before elution continued. All eluted material at this step was collected in fraction 2. Yet another 1 hour pause was made before collection of eluted material in fraction 3. The fractions of high enzyme activity were pooled (approximately 12.5 ml) and concentrated with a Millipore Centriplus YM-30 centrifugal filter unit (Millipore AB, Solna, Sweden) to a volume of 0.9 ml.

(c) Superdex 200 prep grade Gel Filtration Chromatography. Gel filtration of concentrated fractions with α-mannosidase activity from previous Con A Sepharose step was performed with a HiLoad 16/60 Superdex 200 prep grade gel filtration column (1.6 × 60 cm) previously equilibrated in 20 mM Tris-HCl pH 7.5 with 150 mM NaCl. The column was loaded with the ConA Sepharose concentrate at 0.5 ml/min and 2 ml fractions were collected. Fractions of high enzyme activity were pooled (8 ml) and concentrated using a Millipore Centriplus YM-30 centrifugal filter unit to a volume of

0.9 ml. The molecular weight was determined after calibration by using the HMW calibration kit.

Electrophoresis

All electrophoretric equipment and reagent were supplied by Amersham Biosciences, Uppsala, Sweden. Purified α-mannosidase and samples from purification steps were analyzed using Native PAGE and SDS PAGE with 8-25 gradient gels using a PHAST system and staining with Comassie Brilliant Blue. SDS PAGE of the final product was performed under both unreduced (2.5% SDS) and reduced (2.5% SDS with 5% β-mercaptoethanol) conditions.

The molecular weight of the obtained bands was determined by comparing their relative migration distance (Rf) to those of standards of known molecular weight using the LMW Calibration Kit Proteins by plotting the Rf against the logarithms of their molecular weight.

Determination of the position responsible for the α-mannosidase activity in native gels was done by cutting the lanes of a gel run with 8 identical samples into pieces and performing activity tests. Each piece was incubated in eppendorf tubes together with 500 µl of 5 mM pNPMα in 200 mM NaAc pH 4.5 for 2 hours at room temperature followed by 30 minutes at 50ºC. An aliquot of 100 µl of incubation solution and 100 µl of 1 M Na_2CO_3 was mixed in a microtiter plate after which the absorbance at 405 nm was determined. Gel lines not used for activity tests were stained with Comassie Brilliant Blue. By comparing the part of the gel that showed the highest activity with the stained gel the position of the enzyme was identified in the gel. The pI of the pure α-mannosidase was determined by isoelectric focusing (IEF) using a PhastGel IEF 3-9 gel with the Broad pI Kit from Amersham Biosciences. The gel was stained with Comassie Brilliant Blue.

Effect of temperature and thermal stability

Determination of optimum temperature for the α-mannosidase was performed with 5 mM pNPM in 100 mM NaAc-HAc pH 5.0 using incubation temperatures in the interval 20˚C to 70˚C. Thermal stability was investigated after incubation of the

enzyme at 4˚C, 21˚C and 50˚C during 24 hours, 72 hours and several weeks (4˚C). Detection of loss in α-mannosidase activity was performed using standard assay conditions.

Effect of pH

The optimum pH for α-mannosidase activity was determined using 5 mM pNPM in 200 mM NaAc-HAc with pH ranging from pH 3.5 to 6.0 under standard assay conditions.

Substrate specificity

The purified α-mannosidase was investigated for activity towards various substrates at 5 mM; pNP- α-arabinopyranoside, pNP -β-cellobioside, pNP- β-glucopyranoside, pNP-β-fucopyranoside, pNP- α-galactopyranoside, oNP-β-galactopyranoside, oNP-N-acetyl-β-galactosaminide, pNP-N-acetyl, β-galactosaminide, pNP-N-acetyl-β-glucosaminide, pNP- α-mannopyranoside, pNP- β-mannopyranoside, pNP- α-xylopyranoside and pNP -β-xylopyranoside, using standard assay conditions.

Kinetic parameters

The kinetic behavior was examined using standard assay conditions with pNPM in 100 mM NaAc-HaC pH 4.5 except for the addition of 1 mM Zn in the 5 mM pNPM stock solution. All dilutions of enzyme were performed with 0.25 mg BSA. The linearity of the α-mannosidase at fixed substrate and enzyme concentration was tested using reaction times up to 90 minutes. Lineweaver-Burk tests were performed with substrate concentrations ranging from 0.05 mM to 15 mM pNPM at 40˚C and 50˚C.

Effect of EDTA, metal ions and mannose

An aliquot of 750 µl of pure diluted α–mannosidase in 20 mM Tris-HCl pH 7.5 with 0.25 mg/ml BSA was incubated for 2 hours with 250 µl of 0.2 M EDTA containing 0.5 M NaCl pH 7.5. An incubation control was made with 50 mM Tris-HCl pH 7.5 containing 0.5 M NaCl instead of EDTA. The EDTA treated α–mannosidase was desalted using PD10 columns (Amersham Biosciences) previously equilibrated with 100 NaAc-HAc pH 4.5. Elution was performed with 2 ml of equilibration buffer. The

effect of metal ions were studied by incubating 100 µl of desalted sample at room temperature with 10 µl of both 1 and 10 mM of the following salts; $AgNO_3$, $CaCl_2$, $CuSO_4$, $MgCl_2$, $MnCl_2$, and $ZnCl_2$ respectively. The α–mannosidase activity from each incubation was determined using standard assay conditions. The effect of D–mannose was investigated at concentrations ranging from 1 mM to 500 mM.

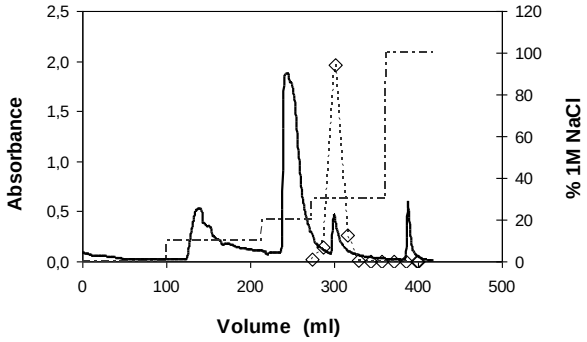
Amino acid composition

The amino acid composition and the content of glucosamine, with the exception of tryptophan, were determined for the purified α-mannosidase at the Amino Acid Analysis Center at the Biomedical Centre of Uppsala University, Uppsala. Lyophilized babaco latex was included as a control of starting material.

Results and discussion

Extraction of α–mannosidase from lyophilized babaco latex was performed in 20 mM Tris-HCl pH 7.5. Protease inhibitors were not used. Preliminary experiments with crude extracts showed high activity of α–mannosidase, but also significant activity of β–glucosidase, Acetyl β–glucosaminidase, Acetyl β–galactosaminidase and cysteine proteases (results not shown). Even though babaco, as recently been described (Kyndt, VanDamme et al.,2007), has a high content of cysteine proteses in its latex, the α–mannosidase in the extract from the lyophilized latex showed no sign of degradation due to protease activity. Initial investigations showed that the α–mannosidase was stable at room temperature for several days with little loss in activity.

The latex extracts were batch adsorbed to Q Sepharose ion exchange resin. This step provided very efficient removal of the majority of the proteins present in the latex extract, while most did not bind to the matrix and could subsequently be washed away by the 20 mM Tris-HCl pH 7.5 equilibration buffer. After packing of the gel in the column (2.6 x 20 cm) washing commenced until baseline was below 0.05 absorbance units. Elution of unwanted material was accomplished by stepwise elution in two elution steps of 0.1 M and 0.2 M NaCl before elution of the α–mannosidase with 0.3 M NaCl (**Figure 1**). The activity yield from elution was excellent with over 96% of the α–mannosidase activity maintained in the eluate pool.

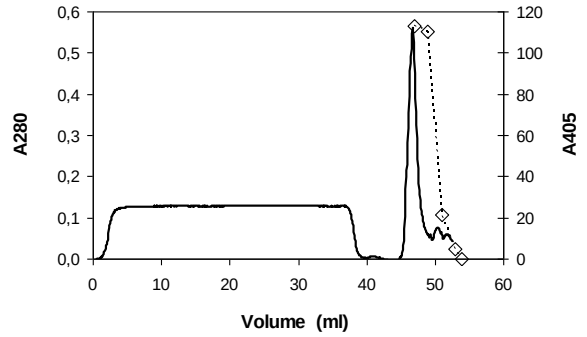


Activity was measured in collected fractions (diamond marks). The salt gradient (combined lines and dots) shows the washing and elution steps with active fractions being eluted at 0.3 M NaCl

Figure 1. Q Sepharose chromatography of babaco latex α-mannosidase

In the second purification step the majority of proteins did not bind to the Con A Sepharose gel, while the α–mannosidase was strongly bound. An earlier attempt with Con A affinity chromatography of α-mannosidase has shown to result in low yields due to the tight binding to the matrix (Waln and Poulton,1987).

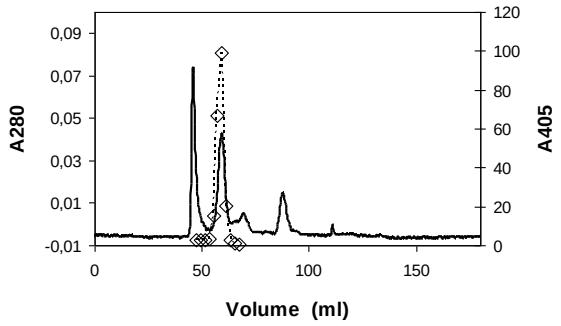
There are also records of successful purification using the same method, but this separation does interestingly not involve the sugar binding site of the lectin (Einhoff, W. and Rudiger,1986). Thus, to improve the yields in these experiments enhanced incubation times in elution buffer for over 8 hours were utilized, resulting in a yield up to this step of over 69 % (**Figure 2**). Fractions showing α-mannosidase activity were pooled and concentrated to be used in the next purification step.



Eluted fractions were tested for activity (diamond marks) and active fractions were subsequently pooled and concentrated.

Figure 2. Con A Sepharose of babaco latex α-mannosidase

The concentrated α-mannosidase from the lectin affinity chromatography was loaded to a Superdex 200 PG gel filtration column as the final chromatographic step and the obtained yield was around 50% (**Figure 3**).



Fractions were measured for activity (diamond marks). Active fractions were pooled and concentrated

Figure 3. Superdex 200 PG gel filtration of babaco latex α-mannosidase

A summary of the purification of the babaco α-mannosidase is shown in **Table I**.

Table I. Purification table of babaco α-mannosidase

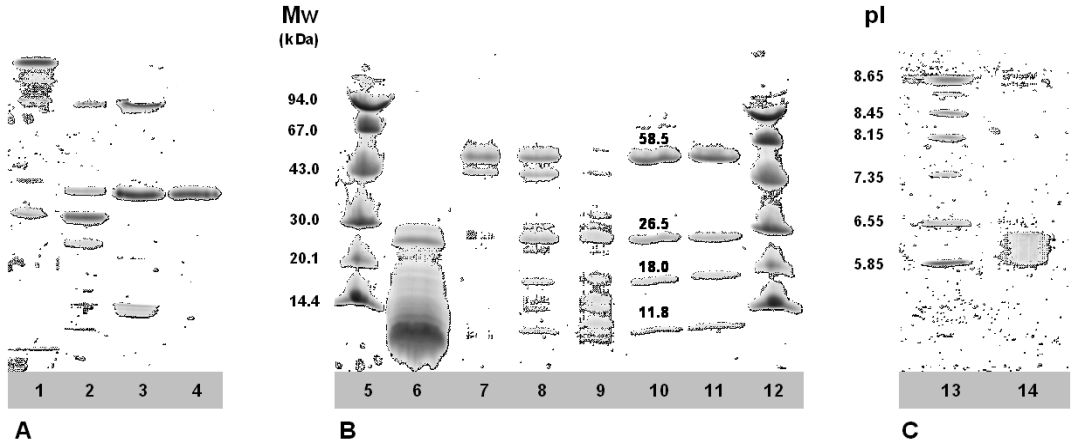
Step	ml	activity	protein conc.	total activity	total protein	specific activity	purification factor	yield
	(ml)	(nkat·ml^{-1})	(mg·ml^{-1})	(nkat)	(mg)	(µkat·mg^{-1})		(%)
CrudeExtract	214.00	3.4	17.48	720.8	3745.620	1.924 ·10^{-4}	1	100,0
Q Sepharose	20.00	34.9	0.28	698.7	5.595	1.249 ·10^{-1}	649	96,9
ConA concentrate	0.50	1005.1	1.69	502.6	0,846	5.943 ·10^{-1}	3088	69,7
Superdex 200PG concentrate	0.03	11904.9	5.21	357.1	0,156	2.283	11863	49,5

The molecular weight was determined to be over 230 kDa from calibration of the column using the Gel Filtration HMW Calibration Kit (Amersham Biosciences). This molecular weight is smaller than that noted from earlier work on papaya (Hartmann-Schreier and Schreier,1986), the closest investigated relative to babaco, but in good agreement with several other purified plant α-mannosidases (Snaith,1975; Paus,1977; Einhoff, Wolfgang and Rüdiger,1988; Niyogi and Singh,1988).

The purity of the α-mannosidase was verified electrophoretically with native PAGE (Coomassie Brilliant Blue staining) and showed one homogenous band (**Figure 4**). The enzymatic identity of the band was also verified by cutting unstained lanes of the same gel into different pieces and performing activity assays on each piece and relating presence of activity with the respective position on the gel (results not shown). Investigation with both unreduced and reduced SDS-PAGE gave identical results revealing four bands. The molecular weights of the subunits were determined and the two largest and most pronounced bands corresponded to molecular weights of 58.5 kDa and 26.5 kDa, respectively, while the two smaller bands correspond to molecular weights of 18 kDa and 12 kDa. There is one report of a heterotetramer mannosidase in *Medicago sativa* (Curdel and Petek,1980) , but due the possible multiplicity of the bands observed the subunit composition could not be established, a situation that also was found for *Prunus serotina Erh*. (Waln and Poulton,1987). It can, however, be concluded that the subunits of the α-mannosidase are not bound to each other by disulfide bridges, as seen by the identical appearance of the lanes with the pure α-mannosidase run in denaturing conditions with and without the presence of mercaptoethanol. This is in accordance with a similar investigation of α-mannosidases of *Phaseolus vulgaris,* which also included a study of dissociation by guanidine hydrochloride followed by sedimentation equlilibrium analysis (Paus,1977).

Even though the multiplicity makes it difficult to establish the subunit composition, based on the molecular weights determined from the individual bands and from size exclusion, an oligomeric structure can be concluded. Thus, an octamer consisting of a total of eight subunits, combined from two each of the identified units of 58.5, 26.5, 18, and 12 is suggested, resulting in a molecular weight of 230 kDa.

Determination of the pI by isoelectric focusing using the Broad pI Kit as a marker showed a diffuse band in the range of pH 5.85 and 6.55.



(A) Native PAGE displaying the steps in the α-mannosidase purification. Coomassie Brilliant Blue was used for protein staining. Lane 1, extraction used for batch adsorption with Q Sepharose; lane 2, concentrated pooled fractions from Q Sepharose; lane 3, concentrated pooled fractions from Con A Sepharose; lane 4, pure α-mannosidase from concentrated Superdex 200 PG fractions. The position on the native PAGE responsible for the α-mannosidase activity was identified by mapping, accomplished by incubation divided gel pieces from lanes identical to lane 4 with pNPMα and performing standard enzyme tests. (B) SDS PAGE stained with Comassie Brilliant Blue. Lanes 5 and 12 are LMW Calibration Kit Proteins. Lane 6, reduced SDS of the extract starting material used for batch adsorption with Q Sepharose; Lane 7, reduced SDS of concentrated pooled fractions from Q Sepharose; lane 8, unreduced SDS of concentrated pooled fractions from Con A Sepharose; lane 9, reduced SDS of concentrated pooled fractions from Con A Sepharose; lane 10, unreduced SDS of pure α-mannosidase from concentrated Superdex 200 PG fractions; lane 11, reduced SDS of pure α-mannosidase from concentrated Superdex 200 PG fractions. (C) Isoelectric focusing with PhastGel IEF 5-8. Lane 13, Broad pI Kit extraction ; lane 14, pure α-mannosidase from concentrated Superdex 200 PG fractions.

Figure 4. Electrophoresis of α-mannosidase

The determined *amino acid composition* of the whole α-mannosidase (**Table II**) was compared to results from previously published plant α-mannosidases from Kidney bean, *Phaseolus vulgaris* (Paus,1977), and two isoforms from Jack bean, *Canavalia ensiformis* (Einhoff, Wolfgang and Rüdiger,1988). All four have very similar composition with only minor deviations, showing high amount of glutamic acid and aspartic acid, valine, serine and leucine. The babaco α-mannosidase has somewhat less content of lysine and threonine. However, differences between the analyzed α-mannosidases from Kidney bean and Jack bean both are also present. The presence of 2% glucosamine points to the degree of glycosylation of the enzyme, which in part could explain the polydisperse pattern found from pI analysis with electrophoresis.

The established amino acid composition of the α-mannosidase was used for a search with the AACompIdent tool at ExPASy Home page in the Swiss-Prot and TrEMBL databases for proteins. No similarity was found in Swiss-Prot, but with TrEMBL the closest entries was found to be that of different clones of α-mannosidase from *Arabidopsis thaliana*, another family of the Brassicales branch and thus more closely related to babaco than kidney bean and jack bean.

Table II. Amino acid composition of babaco α-mannosidase

Residue	babaco	canvalia ensiformis[1]	phaseolus vulgaris I[2]	phaseolus vulgaris II[2]
	%	%	%	%
Ala	6.95	7.65	7.03	7.02
Arg	5.55	4.56	4.49	4.88
Asx[3]	11.96	11.55	12.02	12.30
Cys	trace	n.d	1.02	0.96
Glx[4]	10.65	11.60	11.64	8.33
Gly	6.92	7.37	7.35	7.37
His	2.10	3.25	2.04	2.25
Ile	5.77	3.02	5.31	5.61
Leu	8.64	8.96	9.29	9.59
Lys	5.78	7.65	8.27	8.31
Met	1.08	2.86	trace	trace
Phe	4.51	5.11	4.70	4.75
Pro	4.41	2.75	3.98	4.08
Ser	7.71	8.36	7.66	9.07
Thr	4.68	6.00	5.82	6.10
Trp[5]	xx	Xx	xx	xx
Tyr	5.11	4.90	1.43	1.28
Val	8.17	4.40	7.97	8.10
Glucosamine[4]	2,0	-	-	-

[1]Data obtained from (Einhoff, Wolfgang and Rüdiger,1988). [2]Data obtained from (Paus,1977). [3]Asx is aspartic acid (Asp) and asparagines (Asn). [4]Glx is glutamic acid (Glu) and glutamine (Gln). [5]Tryptophan content was not determined (-) for pure α-mannosidase, thus the total amino acid composition for Canavalia ensiformis and is Phaseolus vulgaris have been corrected with the tryptophan value omitted (xx). The percentage of glucosamine is determined as part of the total amino acid composition including glucosamine. Posts where no residues were detected are marked (n.d). For posts marked with (-) data was not supplied.

From investigations of the thermal stability of the α–mannosidase it was found that storage in 20 mM Tris-HCl pH 7.5 with 150 mM NaCl at 4°C resulted in no detectable decrease in activity during the tested period of more than 2 months. Storage at 21°C for 24 hours did not have any negative effect either, while storage at 50°C for the same time resulted in over 90 % loss in enzyme activity. Even during storage at 21°C for a period of 72 hours there was no observable activity loss (results not shown). The babaco latex α–mannosidase showed a temperature optimum between 50°C and 60°C (**Figure 5**). At 40°C the relative α–mannosidase activity was only 60 % of the optimal, 75 % activity remained at 70°C. Reports have noted optimal temperatures below 40°C (Dey and DelCampillo,1984; Waln and Poulton,1987), but the present results indicate a higher temperature preference similar those found in the more distantly related Solanaceae family (PriyaSethu and Prabha,1997; Suvarnalatha and Prabha,1999).

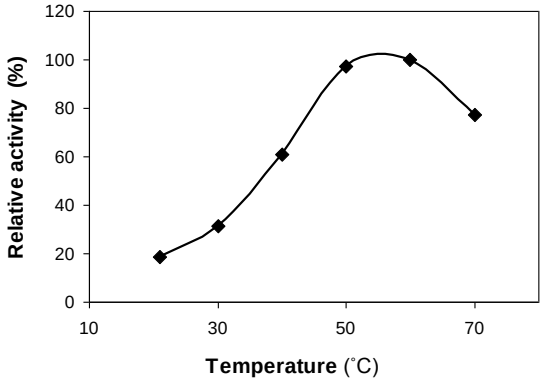
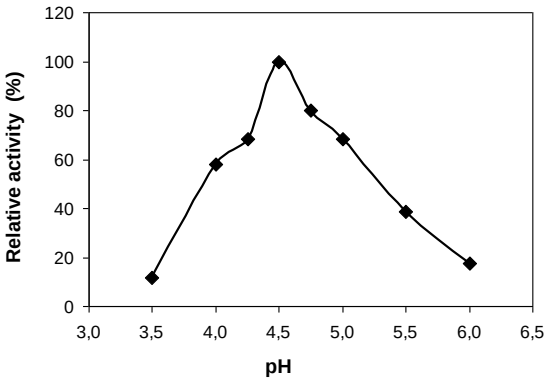


Figure 5. Temperature dependence of the purified α-mannosidase

The determined pH optimum of the α–mannosidase agrees with the majority of earlier published results (Dey and DelCampillo,1984; Waln and Poulton,1987; Einhoff, Wolfgang and Rüdiger,1988; Niyogi and Singh,1988). It is one of the highest noted, with a sharp peak at pH 4.5 and remaining activity at pH 5.0 still higher than at pH 4.0 (**Figure 6**).



The tests for temperature and pH were performed using 5 mM pNPG in 100 mM NaAc incubated for 30 minutes

Figure 6. pH dependence of the purified α-mannosidase

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The K_m was determined to be 1.25 mM using a substrate range from 0.05 mM to15 mM of *p*-nitrophenyl- α-mannopyranoside in 100 mM NaAc-HAc pH 4.5 two different temperatures using 40ºC and 50 ºC. The V_{max} was found to 2.4 μkat·mg^{-1}at 50ºC and 1.94 μkat·mg^{-1} at 40ºC respectively. The values are of the same order of magnitude as to those from other α-mannosidases, but are not directly comparable due to the use of different temperatures and other test conditions (Li,1967; Curdel and Petek,1980; Einhoff, Wolfgang and Rüdiger,1988).

The effect of different metal ions on the α–mannosidase activity was tested by the addition of salts both with and without pretreatment with EDTA for removal of pre-existing ions (**Table III).** Addition of 1 mM ions did not significantly alter the activity, except for Cu^{2+} which reduced the activity to half that of the control. The same pattern was found at 10 mM, but in this case the effect of Cu^{2+} is even more pronounced, leaving only 25% of the activity. Increasing the Zn^{2+} concentration to 1 mM did not increase the activity, but at 10 mM a 50 % increase of activity was observed.

Addition of EDTA strongly reduced the activity, leaving only 36%. This is probably mainly due to the removal of the required Zn^{2+}, as α–mannosidase is a known Zn-dependent enzyme (Snaith,1975). Addition of ions to a concentration of 1 mM after

EDTA treatment did not alter the activity significantly, with the exception of Zn^{2+}, which fully restored the activity. Increasing the concentration of ions to 10 mM did not alter this pattern, with the exception of Cu^{2+}, which further inhibited the enzyme.

The α-mannosidase was very specific for *p*-nitrophenol-α-mannopyranoside and specificity towards all other synthetic *p*-nitrophenol glycoside substrates was below 2%. Testing of product inhibition was performed with different concentrations of mannose. Inhibition was observed above 10 mM. At 100 mM only 50% of the enzyme activity remained and at 500 mM less than 10 % of the activity remained **(Tables IV and V**).

Table III. Effect of metal ions and EDTA on the activity of the α-mannosidase

Addition	Non EDTA treated		EDTA treated	
	relative activity[1] 1 mM %	relative activity[1] 10 mM %	relative activity[1] 1 mM %	relative activity[1] 10 mM %
Without ion	100	100	36	36
Ca^{2+}	99	92	40	39
Cu^{2+}	50	25	39	3
Mg^{2+}	106	110	41	37
Mn^{2+}	73	89	41	71
Zn^{2+}	110	153	134	106

[1] Relative activity of pure α–mannosidase after preincubation for 1 h at 25˚C with different concentrations of metal ions. The activity was measured under standard conditions and is expressed relative to a sample not exposed to any ion.

Table IV. Determination of relative glycosidase activities using various substrates

Substrate[1]	Relative α–mannosidase Activity[2,3] (%)
pNP- α-arabinopyranoside	0.05
pNP -b-cellobioside	0.02
pNP- b-glucopyranoside	0.07
pNP- β-fucopyranoside	0.13
pNP- α-galactopyranoside	0.13
oNP-β-galactopyranoside	0.16
oNP-N-acetyl-β-galactosaminide	0.41
pNP-N-acetyl-β-galactosaminide	0.22
pNP-N-acetyl-β-glucosaminide	0.64
pNP- α-mannopyranoside	100.00
pNP- β-mannopyranoside	0.20
pNP- α-xylopyranoside	1.43
pNP -β–xylopyranoside	0.13

Table V. α–mannosidase product inhibition test with mannose

Mannose concentration (mM)	Relative α–mannosidase Activity [4] (%)
1	106
10	94
50	70
100	51
200	33
500	9

[1]The activity was measured under standard conditions with 5 mM of respective substrate. [2]Activity is expressed as absorbance units measured at 405 nm. [3] Relative activity of pure α-mannosidase for different substrates. Related to pNP- α-mannopyranoside activity (100%) measured under standard conditions with 5 mM of and at respective substrate. [4] Relative activity of pure α–mannosidase in presence of different concentrations of mannose measured under standard conditions and expressed relative to activity in absence of mannose

Conclusions

- The α-mannosidase had the highest activity in babaco latex, and there are no reports in the literature about this kind of enzyme in this fruit.
- The methods used for the purification of the α-mannosidase seem to work successfully because the enzyme was purified and the recovery in the last step was almost 50% which is a good yield. In addition the purified enzyme had a relatively high specific activity 2.283 μkat.mg^{-1}.
- The α-mannosidase of babaco lyophilized latex has been characterized and the optimum pH and temperature was determined to 4.5 and 50ºC respectively, a K_M value of 1.25 mM and a V_{max} value of 2.4 μkat.mg^{-1}, molecular weight around 230 kDa, isoelectric point in the range of 5.85-6.55
- The presence of Zn stimulated the enzyme activity while Cu inhibits the enzyme.
- Regarding the amino acid composition the high content of aspartic acid and asparagine (Asx) was remarkable as well as the content of glutamic acid and glutamine (Glx).

Acknowledgements

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Abbreviations

HP gel, High Performance gel; Con A, Concanavalin A; PG gel, Prep Grade gel; IEF, Isoelectric Focusing; EDTA, Ethylenediaminetetraacetic acid, pNPM, p-nitrophenyl

α-D-mannopyranoside; SDS-PAGE, sodium dodecyl sulphate polyacrylamide gel electrophoresis; HMW, high molecular weight; BSA, bovine serum albumin.

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